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Role of PTEN in Regulation of Cell Migration in Glioma Cells.

IAN MANNION

A thesis submitted to the University of London for the Degree of Master of Philosophy

August 2006

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Declaration

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Abstract

The Phosphatase and Tensin Homologue deleted on Chromosome 10 (PTEN) is a protein and lipid phosphatase whose activity is crucial for tumour suppression. The most notable activity of PTEN is as a phosphatase of the signalling lipid phosphatidylinositol-3,4,5-trisphosphate (PIP_3), and thus as an antagonist of the phosphatidylinositol-3-kinase (PI3K) signalling pathway. Loss of PTEN activity in cells is often an important step in tumourigenesis. In addition to its catalytic site, PTEN also contains a C2 domain, which is able to inhibit cell migration in human glioma cell lines (Raftopoulou et al., 2004). This thesis examines the effect of expression of PTEN and its C2 domain on various aspects of cellular behaviour in human U373 glioma cells. Firstly, it is demonstrated that the PTEN C2 domain shares no significant primary sequence homology with other human proteins, but that its structure is remarkably similar to that of other human C2 domains. Secondly, it is shown that PTEN-C2 is able to interact specifically with a number of inositol phospholipids. Thirdly, phosphorylation at Thr383 is shown to modulate sub-cellular localisation of PTEN. Finally, it is demonstrated that expression of full-length PTEN or the isolated C2 domain is able to perturb the morphology and polarity of U373 glioma cells.

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Chapter 1:

Introduction

1.1. Summary

Cell migration is one of the central topics of interest in biomedical research today. An increasingly sophisticated understanding of the pathology of many prevalent medical conditions has highlighted the significant role played by correctly regulated cell migration in the life both of the cell and of the organism. Notably, aberrant cell migration is of great significance in cancers, where the deregulation of normal cellular processes such as cell adhesion, cell spreading and cell motility allows tumour cells to metastasise to other sites in the organism.

The deregulation of many cellular behaviours observed in tumour cells is often the result of mutations affecting the function of a number of genes, whose activities are known to be of key importance for the proper regulation of cell signalling pathways controlling processes such as the cell cycle, cell adhesion, cell motility and apoptosis. One such gene which is commonly mutated in cancers is the Phosphatase and Tensin Homologue on Chromosome Ten (PTEN). Initially characterised as a dual-specificity protein phosphatase (Li and Sun, 1997;Steck et al., 1997;Li et al., 1997), PTEN rose to a prominent position in

cell signalling when it was demonstrated that it was capable of dephosphorylating the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate, and hence of antagonising the phosphatidylinositol-3-kinase signalling pathway (Maehama and Dixon, 1998). PTEN has since been implicated in controlling a wide range of cellular processes such as cell survival and apoptosis, cell cycle, chemotaxis, cell adhesion and cell migration (Knobbe et al., 2002).

Whilst much interest in PTEN signalling has focussed on its ability to antagonise PI3K, a number of recent studies have shown that PTEN is also able to modulate a number of signalling events independently of its lipid phosphatase activity. In particular, a study by *Raftopoulou et al.* highlighted the ability of the C2 domain of PTEN to down-regulate cell migration in a number of human glioma cell lines, independently of the phosphatase domain (Raftopoulou et al., 2004). This is particularly intriguing, since the precise role of the C2 domain both in the context of the PTEN protein and in the context of the cell remains poorly understood.

This thesis addresses the effects of expression of the PTEN C2 domain on various aspects of cellular behaviour, with the intention of better understanding how it could exert an anti-migratory effect upon human glioma cells.

1.2. Gliomas

Cancer represents perhaps the most pathologically significant group of disorders to affect the populations of developed countries, in terms of both morbidity and mortality. In the UK alone, over 250,000 new cases of cancer are diagnosed yearly, and an estimated one in three people will develop cancer at some stage of their lives. Cancer is also responsible for 26% of all deaths in the UK. Although brain and central nervous system (CNS) cancers are among the least numerically significant cancers, representing the 16th most frequently diagnosed cancers in the UK in 2001 (Cancer Research UK, 2005), they typically

have a poor prognosis, due usually to the aggressively invasive nature of the tumour cells involved (Knobbe et al., 2002). Tumours in the brain and CNS do not typically arise in neuronal cells but in glia, the supporting cells of the CNS. The most common cell types giving rise to gliomas are astrocytes and oligodendrocytes, and all gliomas show an extreme capacity for invasion (Koul et al., 2001). Although they cannot migrate out of the CNS via the lymphatic or circulatory systems, gliomas display a high level of invasion within the CNS. However invasiveness of gliomas does not always correlate with malignancy, and gliomas which are of low clinical grade can often display extensive invasive capacity. The clinical consequences are that 95% of patients undergoing surgery to remove gliomas will present again at a later date, due to infiltration of other tissues (Knobbe et al., 2002). Invading gliomas usually follow a well delineated set of routes along myelinated axons, or basement membrane-like structures of the CNS vasculature. They are extremely motile, due in part to their ability to secrete a variety of matrix metalloproteinases (Koul et al., 2001). They also display an increased proliferative capacity. Hence, an understanding of the migratory processes of glioma cells is essential for the development of treatments. Cell migration and the significance of PTEN, a gene frequently inactivated in gliomas, are discussed in detail below.

1.3. Cell Migration

1.3.1. Migration as a Crucial Cellular Process

Over recent years, many studies have shown that cell migration is a crucial process at all stages of development in most eukaryotic kingdoms and phyla, from plants (Palanivelu and Preuss, 2000) to protozoa (Park et al., 2004) and animals (Ridley et al., 2003). Migration in single-celled organisms, such as the protozoan *Dictyostelium discoideum* can often be relatively simple, where single-celled organisms migrate towards either food sources or mates, for example. However, in relatively large, multicellular organisms such as animals, migration is a much more complex process, and may involve either individual

cells, such as T-cell migration (Ward, 2006) or as large, interconnected groups of cells, such as migration of germ-layers in the embryo (Keller, 2005).

Cell migration is a feature of most stages of the development and life of any particular given organism. In animals, cell migration begins in the earliest stages of development, such as gastrulation, where entire sheets of cells engage in a complex series of movements, to set up the morphogenetic structure of the embryo. At later stages of development, cell migration continues to be of great importance, and various cell types translocate to locations throughout the embryo to continue their development. Even in the adult, cell motility continues to be an essential part of a wide variety of processes, such as immune surveillance (Ward, 2006) and wound closure (Ehrlich, 1988).

In addition to its crucial role in physiological processes, migration is also a feature of many pathological processes. Some pathologies of the immune system, for example, result from defects in one or more of the steps of cell migration, such as Wiskott-Aldrich Syndrome (Ochs and Thrasher, 2006). In addition to conditions resulting from decreased levels of migration, pathologies can also result from unnaturally increased levels of migration. Perhaps the most clinically significant example of this is the process of metastasis, which reflects the transition from a benign to a malignant tumour. Metastasis occurs when malignant tumour cells migrate in a deregulated manner (Nathoo et al., 2005).

1.3.2. The Steps of Migration

Migration is a complex cellular behaviour, consisting of a series of steps, whose precise integration and regulation are essential for net cell translocation (Ridley et al., 2003;Rafelski and Theriot, 2004). In the first of these, a cell receives a cue to initiate migration, the precise nature of which can vary. In response to this cue, the cell undergoes a polarisation event, such that subsequent migratory processes within the cell are directed towards the source of the migration-eliciting cue. Next, the cellular machinery driving protrusion is activated, such

that the cell membrane extends in the direction of migration. Subsequently, adhesion plaques are formed within the area of newly-protruded membrane to stabilise the protrusion and provide further points of traction for the moving cell. Lastly, adhesions at the rear of the cell are dismantled, and the body of the cell undergoes retraction. These steps are cyclical in nature, and the process is repeated throughout cell migration.

1.3.3. Cell Polarisation

In responding to a cue, cells must correctly orient various components so that migration occurs in the direction of the cue. Migration-eliciting cues can be of various types; perhaps the best-characterised examples currently are soluble chemoattractants (Le et al., 2004) or integrin-matrix interactions (Etienne-Manneville and Hall, 1999). Polarisation consists of the re-orientation of several sub-cellular components towards the leading edge of the cell, i.e. the direction of migration. One convenient system which has been employed to examine cell polarisation events during migration is the wound-healing assay (Nobes and Hall, 1999). This assay is carried out in cells which are able to form a confluent monolayer. The monolayer is scratched with a micropipette, such that a wound is created, across which cells may migrate. This is convenient, since wounding a monolayer in this manner induces directed migration, perpendicular to the wound. Microinjection can be used to introduce protein or DNA into the cells at the front of the monolayer, allowing their effects on the polarity of these cells to be readily visualised.

This assay has been exploited to allow various cell biological events to be analyzed, resulting in the definition of a number of criteria for correct cell polarisation. Firstly, the Golgi apparatus is re-oriented such that it sits in front of the nucleus facing the direction of motility. Similarly, the microtubule organising centre (MTOC), the centrosome, is re-oriented such that it is in front of the nucleus in the direction of migration. Lastly, upon wounding, cells produce actin-rich protrusions, which are restricted to the membrane at the

front of the cell, in contact with the wound, and not at the back of the cell, which is in contact with its neighbours (Nobes and Hall, 1999;Etienne-Manneville and Hall, 1999). It has been suggested that MTOC and Golgi reorientation depends on the capture of microtubule (MT) (+)-ends by membrane-components at the leading edge of the cell. This allows vesicles bearing both lipid and protein signalling components essential for motility to be directed towards the front of the cell, in the direction of migration. Much recent work has sought to characterise the molecular mechanisms of these polarisation events, particularly the role of the Rho family of small GTPases.

The Rho GTPase family consists of some 20 proteins and has been implicated in the regulation of a large number of cellular processes. Their regulation of signalling relies upon their ability to bind guanine nucleotides. In the GTP-bound state, Rho GTPases are active, and signal to a large number of target (effector) molecules. However, they also possess GTPase activity, and hydrolyse bound GTP to GDP, resulting in the abrogation of their ability to signal to effectors. GTPases may be reactivated by exchange of GTP for GDP (Hall, 2005). Since both intrinsic GTP/GDP exchange and GTP hydrolysis are slow under physiological conditions, cells facilitate these processes using guanine nucleotide exchange factors (GEFs: (Schmidt and Hall, 2002)) and GTPase-activating proteins (GAPs: (Bernards, 2003)).

The role of the small GTPases Rho, Rac and Cdc42 in establishment of cell polarity in migrating primary rat astrocytes has been studied in some detail (Etienne-Manneville and Hall, 1999). Initial experiments showed that injection of dominant-negative Cdc42, but not dominant-negative Rac or Rho, disrupted the orientation of the MTOC. Further experiments with constitutively active Cdc42 and various Cdc42 GEFs suggested that spatially-restricted activation of Cdc42 was responsible for establishment of polarity. Further analysis revealed that Cdc42 interacted with PAR6, the mammalian orthologue of a *C. elegans* protein implicated in polarised cell division. PAR6 associates with an atypical

protein kinase C (aPKC) via its N-terminus, and this complex is required for MTOC polarity. Thus expression of the N-terminal portion of PAR6 (responsible for interaction with aPKCs), or over-expression of wild-type or kinase-dead PKCζ significantly disrupted proper re-orientation of the MTOC, but interestingly not of protrusions. Finally, cytoplasmic dynein function, which could potentially capture MT (+)-ends at the cell membrane, was shown to be indispensable for MTOC polarity. It was hypothesised that PKCζ activity could be integral to proper dynein function, and has been suggested that MTOC reorientation may occur as a result of pulling forces exerted on the MTOC by dynein anchored at the plasma membrane. The physical association of the Golgi system with microtubules results in its reorientation and thus likely facilitates the transport of secretory vesicles containing new membrane and signalling components such as receptors to the leading edge of the cell (Etienne-Manneville and Hall, 1999;Mellor, 2004)

A further study using rat embryonic fibroblasts (REFs) examined the molecular mechanism underlying the ability of Cdc42 to regulate the polarity of cell protrusions (Cau and Hall, 2005). Inhibition of Cdc42 by injection of the CRIB domain from the Wiskott-Aldrich Syndrome protein (WASp) in the context of a wound-healing assay resulted in the disruption of polarity of cell protrusions. This did not occur after disruption of the PAR6/PKCζ pathway, however inhibition of the Cdc42 effector PAK1, by injection of its autoinhibitory domain, resulted in delocalisation of protrusions to all around the cell's periphery. Immunofluorescence showed that (active) phosphorylated PAK was concentrated at the leading edge of cells, and phosphorylation was dependent on Cdc42 activity. Over-expression of the PAK-interacting Rac GEF, βPIX, or indeed depletion of βPIX expression in cells by RNAi, resulted in an increase in the proportion of cells with no detectable protrusions, and in cells with delocalised protrusions. Finally, βPIX accumulated at the leading edge, and this localisation was dependent on PAK1 activity. Taken together, these results lead to a model (Figure 1.1) in which the wounding of a monolayer results in

activation of Cdc42 and βPIX at the front of the cell (Etienne-Manneville and Hall, 1999). Active Cdc42 activates PAK1 at the front of the cell, which is then able to interact with βPIX and localise it to the front of the cell, although PAK1 has no effect on the activity of βPIX. Active βPIX at the front of the cell is then able to activate Rac in a spatially restricted fashion, which leads to the promotion of cell protrusion.

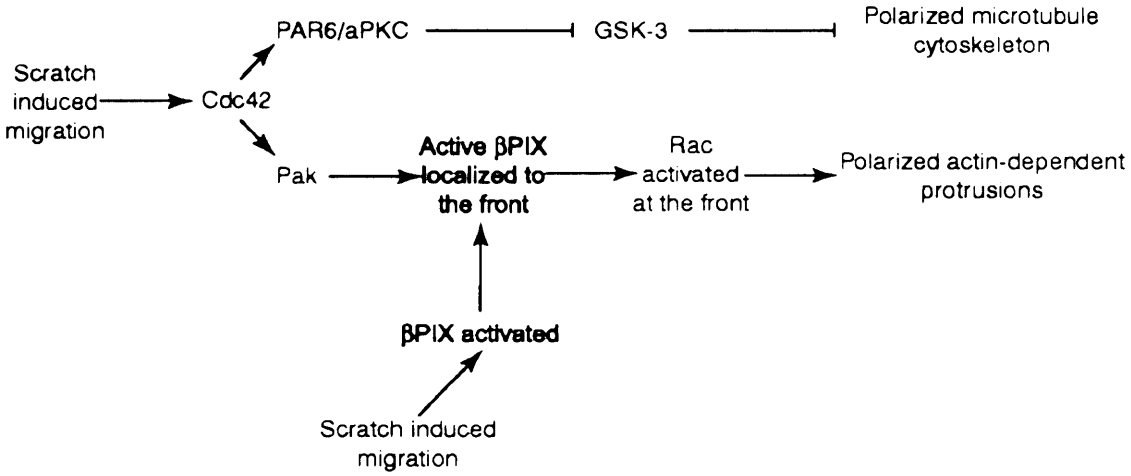


Figure 1.1
Polarity Signalling in REFs. Polarisation of microtubule and actin cytoskeletons is regulated by two distinct, parallel pathways downstream of the small GTPase Cdc42. Microtubule polarity is controlled by the PAR6/aPKC protein complex, while polarity of actin protrusions is controlled by a localised activation of Rac, controlled by the Cdc42 effector Pak (Cau and Hall, 2005).

1.3.4. Membrane Protrusion

Extension and retraction of the cellular membrane represent important ways in which cells interact with their surroundings. Cells possess a repertoire of different types of protrusion, each tailored to the needs of cells of particular types, or of different contexts. Cells such as fibroblasts or keratinocytes extend wide, ruffling protrusions termed lamellipodia, and fine protrusions termed filopodia (Svitkina and Borisy, 1999;Faix and Rottner, 2006). Neurones or glial cells such as oligodendrocytes extend finer, more elongated processes termed growth cones (Mason and Erskine, 2000), whose extremities share some morphological and biochemical similarities to lamellipodia and filopodia. The key factor regulating the formation of cell protrusions is actin dynamics.

Actin is a 42 kDa globular protein of the ATPase family. It is capable of binding either ATP or ADP, and of hydrolysing bound ATP to ADP. Importantly, actin monomers can polymerise to form long, rigid, helical filaments under appropriate salt conditions. These filaments contain an inherent asymmetry, resulting from the asymmetry of the actin monomer, and contain a so-called barbed (+) end and a pointed (-) end. Although actin monomers may freely add to or dissociate from either end of the filament, the barbed end is by far the more dynamic of the two (Pollard, 1986).

In vitro studies have shown spontaneous polymerisation of actin under physiological salt concentrations to be slow (Pollard 1986). Kinetic and thermodynamic modelling has suggested that this is because the formation of actin dimers and trimers, both crucial intermediates for the formation of larger actin oligomers, is thermodynamically unfavourable, and thus rate-limiting (Mullins et al., 1998). Since any cellular motility must be tightly regulated both spatially and temporally and allow rapid responses to migration-eliciting cues, a key feature of any model of cell migration driven by actin polymerisation must be the activation of actin polymerisation by cell signalling factors at the leading edge.

The major regulator of actin polymerisation at the leading edge is thought to be the Arp2/3 complex, a 220 kDa assembly of 7 protein subunits, originally isolated by affinity chromatography on profilin-agarose from extracts of *Acanthamoeba castellani* (Machesky et al., 1994). The subunits of Arp2/3 comprise two actin-related proteins (Arp2 and Arp3), and 5 smaller, novel subunits (ARPC1-5). Arp2 and Arp3 share approximately 40-50% and 30-40% homology with actin respectively, and Arp2/3 expression has been demonstrated to be ubiquitous among eukaryotic phyla (Pollard et al., 2000). The crystal structure of Arp2/3 shows a roughly bilobal morphology, with the two Arp subunits in close proximity (Robinson et al., 2001). This overall

structure is supported by earlier work using electron microscopy and chemical cross-linking experiments (Mullins et al., 1997).

Initial immunofluorescence experiments showed Arp2/3 to be localised prominently to actin-rich regions at the leading edge of lamellipodia (Machesky et al., 1997;Machesky et al., 1994), whilst *in vitro* studies demonstrated how Arp2/3 regulates actin dynamics. Electron microscopy studies showed that Arp2/3 decorates the sides of actin filaments (Mullins et al., 1997), whilst *in vitro* polymerisation experiments showed that Arp2/3 could cap the pointed end of filaments, and also modestly nucleate new filaments (Mullins et al., 1998). Arp2/3 can thus nucleate new actin filaments from the sides of pre-existing filaments at an angle of 70° (Pollard et al., 2000) to generate the dense, dendritic network of filaments found immediately below the lamellipodial membrane of migrating cells (Svitkina and Borisy, 1999).

In fact, the rather modest nature of the nucleating activity of Arp2/3 represents a basal level of activity which can be increased dramatically by Arp2/3 complex-activator proteins, such as those of the WASp/Scar family. N-WASp and Scar1 were first identified as binding partners of Arp2/3 in a 2-hybrid screen (Machesky and Insall, 1998). The mammalian WASp/Scar family consists of 5 members: three Scar (*Dictyostelium* Suppressor of cAMP Receptor mutation) proteins, WASp (Wiskott-Aldrich Syndrome protein) and a related isoform, N-WASp (Neural WASp). All WASp/Scar family members share a largely conserved C-terminus, with binding sites for Arp2/3 and an actin monomer. One attractive mechanism for activation of Arp2/3 is that binding of WASp/Scar family members by Arp2/3 would induce Arp2 and Arp3 to form a steric mimic of an actin dimer; the additional presence of an actin monomer bound to WASp/Scar would allow a cryptic actin trimer to be formed, thus abolishing the thermodynamically unfavourable early steps of actin polymerisation (Pollard et al., 2000;Pollard and Beltzner, 2002).

Whilst the C-termini of WASp/Scar proteins show a high degree of conservation, their N-termini are quite divergent (Millard and Machesky, 2001). However, one common feature is the presence of a region capable of interacting with Rho GTPases, or their targets. This is significant, since WASp/Scar proteins *in vivo* are not usually active, but must be activated by signalling from Rho GTPases. (Higgs and Pollard, 2001;Takenawa and Miki, 2001). In addition to Arp2/3 and members of the WASp/Scar family, many other proteins are implicated in the control of lamellipodial actin dynamics.

Profilin is a small protein which preferentially binds ATP-actin via a site on the actin monomer which would ordinarily be required to bind the pointed end of an existing filament. As a result, profilin-actin is capable of adding only to the barbed end of pre-existing actin filaments. This effectively directs new actin monomers to the more dynamic, barbed end of filaments which are in close proximity to the membrane, and not to the less dynamic, pointed ends (Pollard et al., 2000;Pring et al., 1992). Similarly, although Arp2/3 binds preferentially to ATP-actin filaments (Pollard et al., 2000), which are the youngest, and are thus more likely to be closer to the cell membrane, this preference is not absolute and so the complex may still bind to filaments which are further from the membrane. As a potential source of new filament barbed ends, this would be a waste of actin monomers, since filament initiation far from the cell membrane would not contribute to membrane protrusion. This problem is prevented by the activity of capping protein.

Capping protein (CP) is a heterodimeric protein consisting of an α-subunit, of some 32-36 kDa, coupled with a β-subunit, of approximately 28-32 kDa. CP is widely expressed in cells of the vast majority of eukarotic phyla, including fungi, higher plants and vertebrates. Bioinformatic studies reveal that CP is a unique protein, with no other proteins showing either significant primary sequence or structural similarities (Wear and Cooper, 2004). Both subunits of CP are predominantly α-helical, and interact with each other such that the

resulting complex shows 2-fold rotational symmetry (Yamashita et al., 2003); this is significant, since the barbed end of the actin filament, to which CP binds, also shows such 2-fold rotational symmetry. Although the precise biochemical mechanism of barbed-end binding remains unclear, *in vitro* studies have demonstrated that it is to the barbed end of the actin filament that CP binds. One intriguing mechanism that has been proposed on the basis of structural modelling experiments is the so-called tentacle model, which predicts that protruding α-helices at the C-termini of both CP subunits effectively 'grab' the barbed end of the actin filament and so prevent binding of further actin monomers (Wear and Cooper, 2004). The growth at the barbed ends of actin filaments still occurs at membrane sites, since the phospholipid $PIP(4,5)P_2$, present in membranes, inhibits CP binding (Haus et al., 1991;Heiss and Cooper, 1991). Hence CP activity is of great importance for cell motility, since, together with the activity of profilin, it ensures that free ATP-actin monomers add to filament barbed ends only at sites adjacent to the plasma membrane.

The actin monomers which initially polymerise to form filaments (and hence the new filaments which result from this) are largely in the ATP-bound form. Over time, individual actin monomers hydrolyse bound ATP to ADP, and an inorganic phosphate moiety dissociates. The half-time of ATP hydrolysis is approximately 2 seconds, while the half-time of dissociation of the P_i moiety is around 6 minutes. ADP-P_i- and ADP-actin are therefore markers of filament age, and the change in bound nucleotide also causes a recognisable change in protein conformation (Otterbein et al., 2001). Indeed, various proteins have differing affinities for ATP and ADP filaments and monomers. One such protein is actin depolymerising factor/cofilin (ADF/cofilin)(Maciver et al., 1998;Hawkins et al., 1993). ADF/cofilin has a higher affinity for ADP-actin filaments than for ATP-actin filaments, and so preferentially binds relatively old filaments, which it then severs (Maciver et al., 1998). This is significant, since it promotes recycling of actin monomers from old filaments. After ADP-

dissociation and ATP-binding followed by exchange of cofilin for profilin, recycled actin monomers can be incorporated at filament barbed ends.

Although there are many other proteins involved in the control of actin-driven protrusion, those detailed here can be used *in vitro* to reconstitute the motility of the intracellular parasite *Listeria monocytogenes*, which moves through the cell by usurping the cell's own actin polymerisation machinery (Pizarro-Cerda and Cossart, 2006). As a result, it has been possible to generate a dendritic nucleation model (Fig. 1.2) of actin-driven protrusion in lamellipodia and other, analogous motile organelles of the cell. This appears to conform well to the experimental evidence gained thus far (Pollard et al., 2000), and suggests that the proteins detailed here form a minimum set of components required for the conversion of actin dynamics into controlled cell motility.

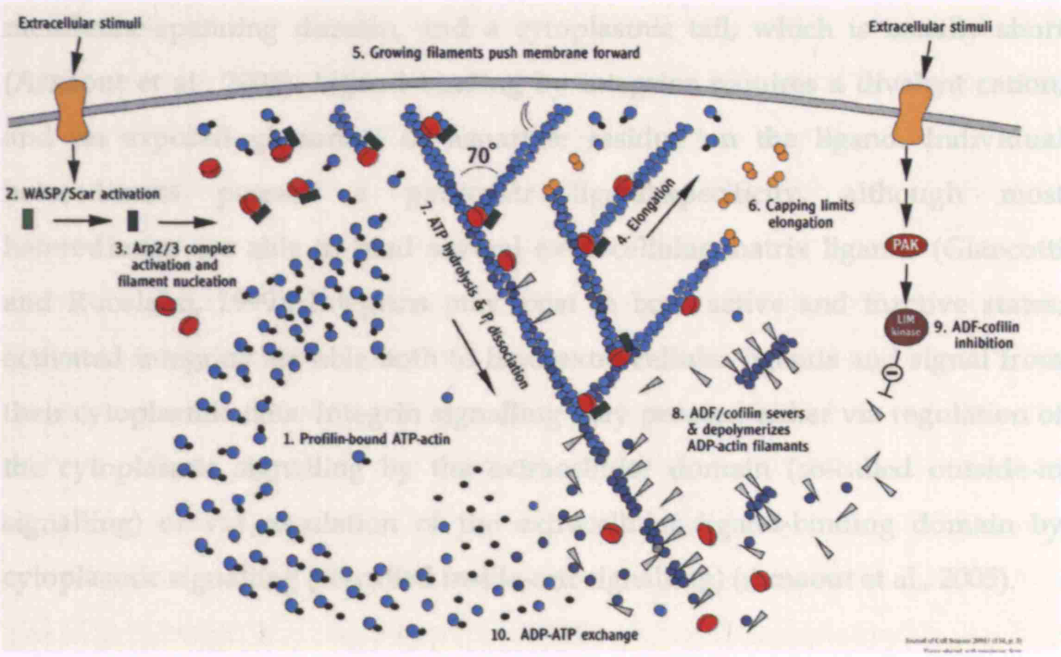


Figure 1.2
The Dendritic Nucleation Model of Actin-driven Protrusion. A model can be constructed from studies, showing how control of actin dynamics can account for membrane protrusion at motile organelles such as lamellipodia. ATP-actin; ADP-actin; Profilin; ADF/Cofilin; Arp2/3; Capping Protein; Inactive WASp/Scar; Active WASp/Scar.

1.3.5. Focal Adhesions

Following the initial stages of actin-driven membrane protrusion, newly extended membrane must be stabilised through adhesion to the substratum. Such adhesion usually occurs in the form of large, multi-protein complexes termed focal adhesions (Ridley et al., 2003). Focal adhesions are among perhaps the most complex protein aggregates in the cell, and so an account of their formation, composition and regulation remains incomplete. However, recent studies have begun to elucidate significant aspects of focal adhesion behaviour.

One particularly well-studied subset of cell-matrix adhesions involve integrins, a large family of proteins, consisting of a heterodimer of α– and β–subunits. In mammals, 24 integrins have been described, consisting of pairings of 18 α-subunits and 8 β-subunits (Giancotti and Ruoslahti, 1999). Each subunit consists of an N-terminal, globular 'head', which serves as a ligand-binding site, a membrane-spanning domain, and a cytoplasmic tail, which is usually short (Arnaout et al., 2005). Ligand binding by integrins requires a divalent cation, and an exposed glutamate or aspartate residue on the ligand. Individual heterodimers possess a particular ligand-specificity, although most heterodimers are able to bind several extra-cellular matrix ligands (Giancotti and Ruoslahti, 1999). Integrins may exist in both active and inactive states; activated integrins are able both to bind extra-cellular ligands and signal from their cytoplasmic tails. Integrin signalling may proceed either via regulation of the cytoplasmic signalling by the extracellular domain (so-called outside-in signalling) or via regulation of the extracellular ligand-binding domain by cytoplasmic signalling (so-called inside-out signalling) (Arnaout et al., 2005).

It is becoming increasingly clear that the blanket term 'focal adhesion' actually refers to a variety of cellular structures, and it is possible to differentiate perhaps four sub-types of 'focal adhesion' (Wozniak et al., 2004): focal complexes, focal adhesions, fibrillar adhesions and 3D matrix adhesions. Of these, the most extensively studied are focal complexes (FCs) and focal

adhesions (FAs). FCs are small, nascent adhesive complexes found at the periphery of migrating cells. There is evidence that they are likely regulated by Rac and Cdc42 (Nobes and Hall, 1995), and that they can mature into FAs, which are larger and regulated by Rho (Hotchin and Hall, 1995;Ridley and Hall, 1992). Staining for markers of adhesions such as β_3-integrins or phosphotyrosine (pY) reveals that size and location are two of the most obvious properties differentiating FCs and FAs; FCs are ordinarily small, punctate structures found almost immediately behind the leading edge of the lamellipodium of a motile cell, whereas FAs are larger structures, usually situated further back from the leading edge. These two sub-populations of adhesions also show different dynamics, with FCs being very much transient structures, which may either dissolve after several minutes, or elongate and mature into FAs (Zaidel-Bar et al., 2003).

A relatively recent study sought to further characterise the molecular composition of FCs and FAs, in an effort to better understand their properties and their various contributions to the phenomenon of cell adhesion, and deserves particular mention here (Zaidel-Bar et al., 2003). The authors observed adhesion dynamics in porcine aortic endothelial cells using a wound-healing system, in which confluent monolayers of cells were scratched, in order to induce migration in those cells on the edge of the resulting wound. Staining for pY and use of a GFP-β_3-integrin fusion protein revealed firstly that the adhesions showed the size and positional distributions described above, based on the localisation of GFP-β_3-integrin, and secondly that pY and GFP staining colocalised in over 80% of cases, suggesting that pY is a reliable marker for FAs and FCs. Subsequently, the authors proceeded to conduct experiments in which cells in a wound-healing assay were co-stained for pY and nine proteins known to exist in the context of FCs/FAs, namely: β_3-integrin, talin, paxillin, vinculin, focal adhesion kinase (FAK), VASP, α-actinin, tensin and zyxin. pY staining was considered a reliable marker of adhesions, and indeed small, punctuate areas of pY staining (i.e. FCs) occurred immediately behind the leading edge,

while larger, elongated areas of staining occurred further back (i.e. FAs). The degree of localisation of the various adhesion proteins in FCs and FAs in defined, lamellipodial regions of cells was measured by analysis of microscopy data. The authors determined that all of the proteins, with the exception of tensin, were found in greater than 80% of FAs in the leading lamellipodium of motile cells, whereas the degree of localisation of most of these proteins (with the exception of β_3-integrin) in FCs was in general much lower. Nonetheless, only zyxin and tensin were found to be almost absent from FCs, and FCs were found to be composed of talin, paxillin, vinculin, FAK, VASP and α-actinin. Hence, the authors determined that tensin and in particular zyxin were reliable markers of mature FAs. In a final experiment, the authors confirmed earlier results regarding the regulation of FCs and FAs by the Rho family of GTPases (Hotchin and Hall, 1995;Nobes and Hall, 1995;Ridley and Hall, 1992). Transfection of cells with constitutively active Rac1 resulted in formation of zyxin-negative adhesions, deemed to be FCs, at the cell periphery. Treatment of cells with the Rho-Kinase (ROCK) inhibitor Y27632 resulted in an increased number of FCs at the cell periphery, and a complete depletion of FAs. This finding is interesting, since the depletion of FAs by inhibitors of ROCK and myosin light chain kinase (MLCK), both of which are implicated in control of acto-myosin contractility, suggests that such contractility could be important for maturation of FCs into FAs.

FAs play an important role in cell behaviour (Mitra et al., 2005), and so the interaction of acto-myosin stress fibres with integrin-dependent cell-matrix adhesions, which may prompt the maturation of transient FCs into more stable FAs (Zaidel-Bar et al., 2003), is significant. Possibly the most significant adhesion protein to mediate this interaction is talin. Talin is a relatively large (2541 amino acids) protein, and two talin genes exist in mammals; *Tln1* is ubiquitously expressed, but *Tln2* shows a more restricted pattern of expression (Critchley, 2005). Structural data suggest that talin is a dimer (Isenberg and Goldmann, 1998), and that monomers consist of an N-terminal globular head

and a C-terminal rod-like region (Critchley, 2005). The globular head contains a wide variety of signalling domains, including a FERM (four-point-one, ezrin, radixin, moesin) domain, is capable of binding various interactors, including the cytoplasmic tails of β-integrins (Garcia-Alvarez et al., 2003), FAK (Borowsky and Hynes, 1998)and actin (Lee et al., 2004). The rod is also capable of interacting with actin, integrins and vinculin (Critchley, 2005). The talin head domain is capable of interacting with phospholipids (Seelig et al., 2000), and talin is known to activate integrins (Tadokoro et al., 2003). This is perhaps suggestive of a model in which local production of phospholipids in lamellipodia, for example, could result in recruitment of talin to the cell membrane, with concomitant activation of integrins and recruitment of other adhesion proteins to form FCs (Critchley, 2005). This model provides, in talin, an insight into a potential physical linkage between integrin-dependent cell-matrix adhesions and the actin cytoskeleton. Talin is a component of FCs (Zaidel-Bar et al., 2003), and hence talin-mediated interactions between FCs and the actin cytoskeleton may contribute to signalling prompting the maturation of FCs into FAs, as previously detailed.

Another protein important for adhesion dynamics is FAK, which is recruited to adhesions shortly after integrin clustering (Carragher and Frame, 2004). Recruitment of FAK to adhesions is followed by auto-phosphorylation on Tyr397, with the result that FAK is then able to recruit other molecules such as the tyrosine kinase Src via binding of its SH2 domain to phosphotyrosine residues, and Src is able to further phosphorylate tyrosine residues on FAK, which serve as further docking sites for focal adhesion proteins (Carragher and Frame, 2004;Mitra et al., 2005). Regulation of phosphorylation of FAK at different sites can modulate FAK dynamics; phosphorylation of Tyr397 is associated with integrin clustering, or activation of FAK by other experimental means (Carragher and Frame, 2004), whereas phosphorylations in the so-called focal adhesion targeting (FAT) domain result in the exclusion of FAK from focal adhesions (Liu et al., 2002). As with most components of cell motility signalling

pathways, focal adhesions must be dynamically assembled and disassembled for the cell to respond flexibly to migratory signals. Similarly, focal adhesions at the rear of the cell must be turned over for this region to retract as the cell moves forwards. Several pathways of focal adhesion turnover have been described, and FAK is thought to be a key regulator of this process. Firstly, recruitment of Src to adhesions by FAK leads to focal adhesion turnover, possibly through phosphorylation of FAK at Tyr925 (Wozniak et al., 2004;Mitra et al., 2005;Liu et al., 2002). Secondly, the calcium-dependent protease calpain has been implicated in focal adhesion turnover, as it is able to cleave a number of focal adhesion proteins, such as α-actinin, Src, FAK, paxillin and talin (Friedrich and Bozoky, 2005;Carragher and Frame, 2004).

1.4. The PTEN Tumour Suppressor

1.4.1. Introduction

The PTEN tumour suppressor was discovered in 1997 by three groups (Li et al., 1997;Li and Sun, 1997;Steck et al., 1997); two of these (Li et al., 1997;Steck et al., 1997) had begun an explicit search for a tumour suppressor gene on chromosome 10, prompted by the finding that advanced cancers in humans frequently show mutations at the 10q23 locus. Indeed, loss of heterozygosity at the 10q23 locus is present in some 70% of glioblastomas, and approximately 60% of advanced prostate cancers (Li et al., 1997). Various other pieces of experimental evidence supported the idea that a potential tumour suppressor was located at this chromosomal site, chief among these being that reintroduction of a wild-type chromosome 10 suppressed the tumourigenicity of glioblastoma cells (Hsu et al., 1996).

Each of the three discovering groups assigned a different name to the gene. *Li et al.* termed it Phosphatase and Tensin Homologue, deleted on Chromosome 10 - PTEN (Li et al., 1997) - whilst *Steck et al.* named the gene Mutated in Multiple Advanced Cancers 1 - MMAC1 (Steck et al., 1997) - recognising its significant role as a tumour suppressor. Finally, *Li and Sun* employed yet another name to

reflect the regulation and expression of the protein: Transforming Growth Factor-regulated and Epithelial Cell-Enriched Phosphatase 1 - TEP1 (Li and Sun, 1997). PTEN is the name currently most often employed in the literature.

Work in other systems has now shown that PTEN is ubiquitously expressed across all major eukaryotic phyla including fungi, such as yeast (Maehama et al., 2001), protozoa (*Dictyostelium discoideum* e.g. (Iijima and Devreotes, 2002)), plants (*Arabidopsis thaliana* (Gupta et al., 2002)), invertebrates (e.g. *Drosophila melanogaster* (Goberdhan et al., 1999)), and mammals. In humans, the PTEN protein is 403 amino acids long, and the PTEN N-terminus shares a high degree of homology with the protein tyrosine phosphatase family and the cytoskeletal protein tensin (Li et al., 1997). At the extreme N-terminus, there exists a short, positively-charged stretch of amino acids which function as a lipid-binding domain, and which are thought to be essential for membrane-binding and tumour suppressor function (Walker et al., 2004). This is followed by the phosphatase domain. Although this was initially classified as a protein tyrosine or dual-specificity phosphatase on the basis of homology (Li et al., 1997;Myers and Tonks, 1997), later studies demonstrated that the major physiological substrate of PTEN is in fact a phospholipid: phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3, PIP_3, (Maehama and Dixon, 1998)). This was significant, since no such enzymatic activity had been demonstrated before this time. The C-terminal globular domain, initially thought to be a tensin/auxillin-like domain, is a C2 domain (Lee et al., 1999). The C2 domain is followed by a flexible, unstructured tail containing several phosphorylatable serine and threonine residues. The phosphorylation state of PTEN affects its function and regulation (Vazquez et al., 2000). Finally, a C-terminal PDZ-binding motif allows PTEN to interact with various PDZ-containing proteins (Wu et al., 2000a;Wu et al., 2000b). Orthologues of human PTEN conform broadly to this description of the domain structure of mammalian PTEN, with some exceptions (Figure 1.3). *Drosophila* PTEN exists in three splice variants, only one of which contains a PDZ-binding motif (Smith et al., 1999).

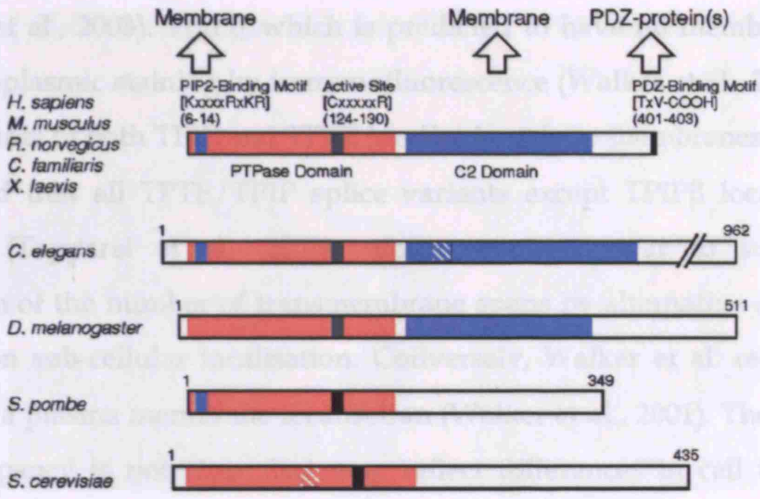


Figure 1.3
PTEN Orthologues. PTEN orthologues have been characterised across a wide range of eukaryotes including mammals (e.g. *H. sapiens, M. musculus*), amphibians (*X. laevis)*, nematodes (*C. elegans*), *Drosophila melanogaster*, and yeasts (*S. cerevisiae, S. pombe*). Most orthologues characterised thus far contain both phosphatase (pink) and C2 (blue) domains; the yeasts *S. cerevisiae* and *S. pombe* are notable exceptions to this, with no C2 domain having yet been described (Maehama et al., 2001).

1.4.2.The Human PTEN Gene Family

The human genome sequence contains a wider family of genes related to PTEN, usually termed the Transmembrane Phosphatase with Tensin Homology (TPTE) family (Tapparel et al., 2003). There exist two related sub-families: TPTE and the TPTE- and PTEN-homologous Inositol lipid Phosphatase (TPIP) and several splice variants, giving rise to TPIPα, -β, -γ and -δ, as well as TPTEα, -β and -γ, have been identified. Northern blot analysis reveals that TPTE splice variants are restricted to the testis, while TPIP, while abundant in testis, is also found at lower levels in brain and stomach (Walker et al., 2001;Chen et al., 1999;Tapparel et al., 2003). At least one of these homologues, TPIPα, possesses

phospholipid phosphatase activity, but the physiological significance of this is not clear (Knobbe et al., 2002;Tapparel et al., 2003). Bioinformatics predicts that each of these proteins contains membrane-spanning domains, with four membrane-spans in each of the TPTE splice variants. In TPIP splice variants, alternative splicing affects the predicted number of membrane spans, for TPIP-γ, -α, -δ and -β four, three, two and no transmembrane spans respectively (Tapparel et al., 2003). TPIPβ, which is predicted to have no membrane-spans, shows cytoplasmic staining by immunofluorescence (Walker et al., 2001). Other splice variants of both TPIP and TPTE localise largely to membranes. *Tapparel et al.* reported that all TPTE/TPIP splice variants except TPIPβ localise to the ER/Golgi (Tapparel et al., 2003), which would appear to suggest that modulation of the number of transmembrane spans by alternative splicing has no effect on sub-cellular localisation. Conversely, Walker et al. reported that TPTEγ has a plasma membrane localisation (Walker et al., 2001). The reason for this discrepancy is not clear, but may reflect differences in cell types used. Currently, no convincing cellular role has been proposed for any of these proteins (Tapparel et al., 2003).

1.4.3. Structure and Function of the PTEN Tumour Suppressor

The crystal structure of PTEN was solved in 1999 (Lee et al., 1999), and this provided many important insights into its function and regulation. PTEN contains two independently folding globular domains: the N-terminal phosphatase domain and the C-terminal C2 domain. Further analysis of the X-ray crystallography results and of proteolytic digestion experiments demonstrated the existence of three unstructured loops: a 7 amino acid loop at the extreme N-terminus, a 24 amino acid loop between the phosphatase and C2 domains, and finally a 49 amino acid loop at the extreme C-terminus (Fig. 1.4)

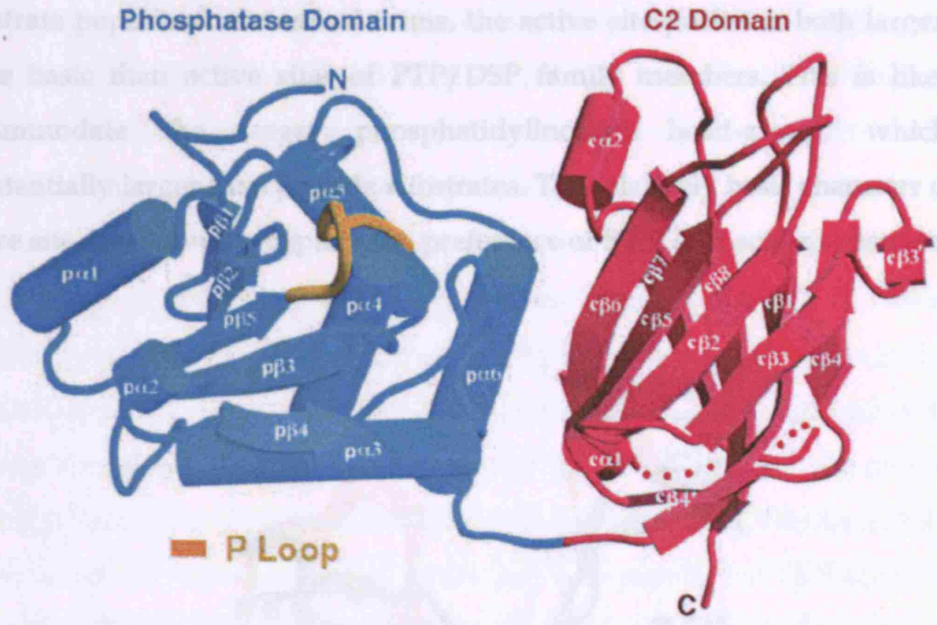


Figure 1.4
Crystal Structure of the PTEN Tumour Suppressor. X-ray crystallography determined that the PTEN protein is composed of two independently-folding globular domains. The largely α-helical phosphatase domain (blue) occurs towards the N-terminal of the protein, and contains the P-loop (orange); the C2 domain (pink), consisting largely of β-sheets, is situated towards the C-terminal. These two globular domains are separated by a flexible linker, and additional flexible tails (not included in the crystal structure) are found at the N- and C-termini. The residues Cys124 and Gly129, mutations of which are frequently used in studies, are situated in the P-Loop – see Fig. 1.5 (Lee et al., 1999).

1.4.3.1. The Phosphatase Domain

The phosphatase domain consists of a central β-sheet which is surrounded by α-helices. In the centre of this are the active site residues (123-30) (Fig. 1.5), including an HCXXGXXR sequence common to members of the protein tyrosine phosphatase (PTP) and dual-specificity phosphatase (DSP) families.

Outside of this signature motif, there is little homology with members of these families, and indeed the active site of PTEN itself shows many striking differences from those of the PTP/DSP families, both in terms of structure and function. In the first place, although PTEN has been shown to dephosphorylate various proteins *in vitro*, such as FAK (Tamura et al., 1998), this activity is weak compared to other protein phosphatases, and requires an extremely acidic substrate peptide. In structural terms, the active site pocket is both larger and more basic than active sites of PTP/DSP family members. This is likely to accommodate the larger phosphatidylinositol head-group, which is substantially larger than peptide substrates. The relatively basic character of the active site also serves to explain the preference of PTEN for acidic substrates.

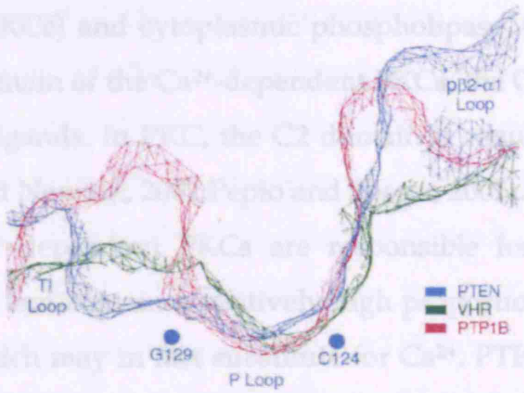


Figure 1.5
Active Site Structures of PTEN and Other Phosphatases. In comparison with the active sites of other phosphatases such as VHR (green) and Protein Tyrosine Phosphatase 1B (PTP1B – pink), that of PTEN is relatively large, and this structural feature facilitates the entry of large, phosphatidylinositol substrates. Marked are the G129 and C124 residues, mutation of which leads to a loss of either protein phosphatase (G129E) or total phosphatase (C124S) activity (Lee et al., 1999).

Within the active site, two residues in particular have proved important for functional dissections of the importance of PTEN in various cellular signalling pathways. The first is C124, a cysteine residue essential for catalysis; mutation to serine (C124S) is sufficient to completely abolish catalytic activity of the protein (Tonks and Myers, 1999). The second is G129, a glycine residue whose

replacement by an acidic glutamate residue (G129E) provides an electrostatic hindrance with the D5 phosphate group of the phosphatidylinositol head group; the catalytic consequence of this is the abrogation of lipid phosphatase activity whilst protein phosphatase activity is unaffected (Maier et al., 1999).

1.4.3.2. The C2-Domain

The C2-domain lies toward the C-terminus of the protein and, though separated from the catalytic domain by a 24-residue flexible loop, maintains interactions with this over a large interface. In contrast to the phosphatase domain, the C2-domain consists predominantly of β-sheets, with two antiparallel β-sheets surrounding two short α-helices. The PTEN C2 domain exhibits a Type II C2 fold, similar to that of the C2 domains found in phospholipase Cδ1 (PLCδ1), protein kinase Cδ (PKCδ) and cytoplasmic phospholipase A2 (cPLA2). Unlike the archetypal C2 domain of the Ca^{2+}-dependent PKCs, the C2 domain of PTEN does not bind Ca^{2+}-ligands. In PKC, the C2 domain is required for membrane binding (Nalefski and Newton, 2001;Pepio and Sossin, 2001). However, the CBR loops which in Ca^{2+}-dependent PKCs are responsible for Ca^{2+}-binding are present in PTEN-C2, and contain a relatively high proportion of basic, solvent-exposed residues which may in fact substitute for Ca^{2+}. PTEN-C2 does possess an affinity for phospholipid vesicles of phosphatidylcholine/serine at least *in vitro*, and, although there is some discussion about this in the literature, the major function of the C2 domain of PTEN is held to be membrane binding and/or the correct positioning of the active site with respect to substrate. *Georgescu et al.* find that whilst artificial myristoylation of wild type PTEN serves to increase its tumour suppressor function, this is not sufficient to rescue activity in a C2 mutant which is defective in membrane binding (Georgescu et al., 2000). The interpretation given is that the precise manner in which C2 binds lipid membranes is important for the correct positioning of the active site of the phosphatase domain respective to its substrate. However, another study addressed the membrane-binding role of the C2 domain by measuring the binding constant (K_d) of recombinant PTEN mutants binding to artificial

vesicles mimicking the inner plasma membrane (Das et al., 2003). The affinity of the isolated C2 domain for lipid vesicles was some 30-fold lower than that of full-length, wild-type PTEN, suggesting that the C2 domain does not drive membrane-recruitment of PTEN. Whatever the precise role of this domain in membrane targeting, mutants in the C2 domain are able to modulate the *in vitro* catalytic activity of the phosphatase domain. Indeed approximately 50% of PTEN point mutations in tumours lie within the C2 domain, suggesting that its structural integrity is important for the catalytic activity of the full-length protein (Georgescu et al., 2000;Georgescu et al., 1999).

1.4.3.3. The N-terminal Lipid-Binding Motif

The precise mechanisms by which PTEN is recruited to membrane sites have been explored in another study in which a highly-conserved, polybasic, putative $PI(4,5)P_2$ binding site at the extreme N-terminus of PTEN was characterised (Walker et al., 2004). Experiments centred on the biochemical characterisation of the PTEN-K13E mutant found in non-Hodgkin's malignant lymphoma. In contrast to most tumour-derived PTEN mutants, K13E maintains its catalytic activity *in vitro* and is comparable to wild-type PTEN. Bioinformatic analysis had predicted the existence of a $PI(4,5)P_2$ binding-site at the N-terminus (Maehama et al., 2001) and *in vitro* studies confirmed that the inclusion of anionic lipids, and in particular $PI(4,5)P_2$, within artificial lipid vesicles greatly enhanced catalytic activity towards vesicular lipid substrates (McConnachie et al., 2003). This supports the hypothesis that a $PI(4,5)P_2$-binding domain is present within PTEN and that it is important for function. Interestingly, the K13E mutant did not respond to addition of $PI(4,5)P_2$ to lipid vesicles. Furthermore, transient transfection of PTEN-K13E into the PTEN-null glioma cell line U87MG produced none of the effects typically associated with the re-introduction of a catalytically active PTEN into cells, such as decreased phosphorylation levels of PKB/Akt. Inclusion of a myristoylation site on PTEN-

K13E, on the other hand, rescued catalytic activity *in vivo* (Walker et al., 2004). Taken together, these results explain the preference of PTEN for acidic membranes (Das et al., 2003), and suggest that the N-terminal PI(4,5)P_2-binding motif of PTEN, centred around residue Lys13, is essential for membrane binding and cellular function of the tumour suppressor (Walker et al., 2004).

1.4.3.4. The C-terminal Flexible Tail

The precise structural contribution of the flexible tail downstream of the C2 domain of PTEN is not known, since it was not present within the recombinant protein used to generate the crystal structure of PTEN (Lee et al., 1999). Functionally, however, this tail sequence, consisting of residues 353-399, is of considerable importance for the regulation of PTEN, since it contains numerous phosphorylatable serine and threonine residues. Modulation of these phosphorylation sites can have profound consequences for the stability and/or catalytic function of PTEN, which are discussed later.

1.4.3.5. PDZ-binding Motif

The C-terminus of PTEN encodes a PDZ-binding motif. This can interact with PDZ domains present in a variety of proteins (Valiente et al., 2005;Wu et al., 2000b). PDZ domains were initially named after the proteins **P**sd95, **D**lg and **Z**O-1, and are widespread, occurring across most, if not all, eukaryotic phyla, including the nematode *C. elegans*, *D. melanogaster*, humans, various plants and, to a limited extent, yeasts (Nourry et al., 2003). Three families of PDZ-containing proteins have been defined. The first family comprises proteins consisting entirely of PDZ domains, such as syntenin (Sarkar et al., 2004). The second family are the Membrane-Associated Guanylate Kinases (MAGUKs), such as Dlg (Humbert et al., 2003) or MAGI-2 (Tolkacheva et al., 2001) and the final family includes non-MAGUK proteins which are comprised of PDZ and non-PDZ domains, for example Scribble (Humbert et al., 2003).

The ligands of PDZ-domains are proteins with PDZ-binding motifs, which are C-terminal sequences of three or four amino acids. Three classes of these motifs have been defined, and these have varying binding specificities (Table 1.1). The PDZ-binding motif of PTEN, with the sequence -TKV, is a Class I motif. Various PDZ domain-containing proteins have been identified as interactors of PTEN, including the MAGUKs MAGI-1/2/3 (Tolkacheva et al., 2001;Wu et al., 2000a;Wu et al., 2000b;Kotelevets et al., 2005), Dlg and the Microtubule-associated Serine/Threonine Kinase-205 (MAST205 - (Valiente et al., 2005). The significance of these interactions will be discussed more fully below.

Class	C-terminal sequence	Interacting protein	PDZ domain-containing protein
Class I			
X-S or T-X-V	E-S-D-V	NMDAR2A, B	PSD-95 (PDZ2)
	E-T-D-V	Shaker-type K^+ channel	
	D-S-W-V	p0071	Erbin
		δ-catenin, ARVCF	
X-S or T-X-L	D-S-S-L	β_2-adrenergic receptor	NHERF or EBP50
	Q-T-R-L	GKAP	Shank pr ProSAP
Class II			
X-Ψ-X-Ψ	E-Y-Y-V	Neurexin	CASK
	E-F-Y-A	Syndecan	CASK, syntenin
	S-V-E-V	EphB2	PICK1
	D-V-P-V	ErbB2	Erbin
Class III			
X-D or E-X-Ψ	V-D-S-V	Melatonin receptor	nNOS
	G-E-P-L	KIF17	mLIN10 or Mint1 or X11

Table 1.1: Sequences and ligands of Class I, II and III C-terminal PDZ-binding motifs. (Modified from Nourry et al., 2003).

1.4.4. PTEN - an antagonist of PI3K Signalling

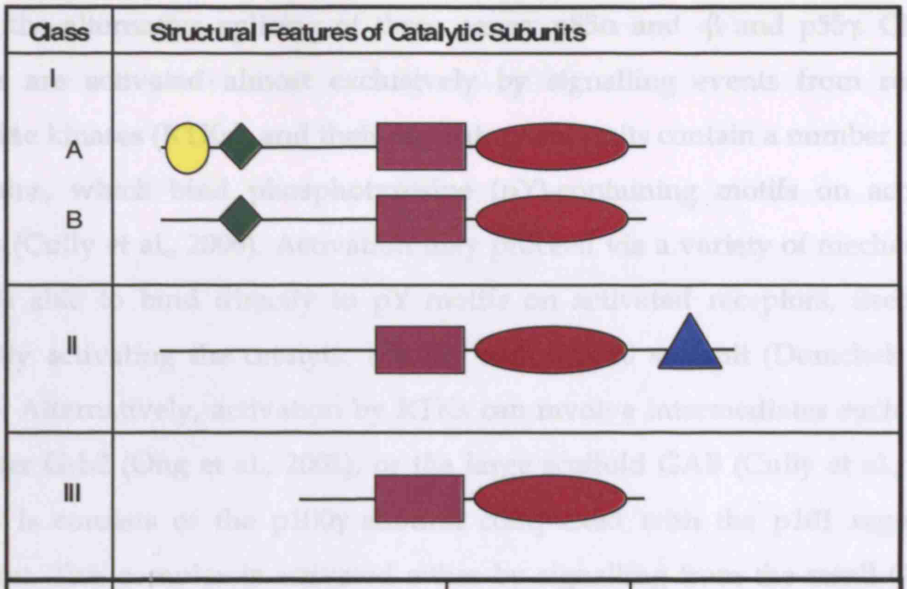
PTEN was originally characterised as a protein phosphatase and indeed it is able to dephosphorylate protein substrates such as FAK *in vitro* and *in vivo* (Tamura et al., 1998). However, a major breakthrough occurred in 1999, with the finding that the major physiological substrate of PTEN is the lipid second messenger PI(3,4,5)P_3 (Maehama and Dixon, 1998). This is of some significance, since PIP_3, a product generated by phosphatidylinositol-3-kinase (PI3K) activity is a potent signalling molecule which promotes cell survival and growth, and

which plays an important role in chemotaxis (Iijima et al., 2002). Most studies now focus on this aspect of PTEN signalling, which is considered to be the most significant cellular role of the protein.

1.4.5. Phosphatidylinositol-3-Kinases (PI3Ks)

PI3Ks are a family of enzymes that can phosphorylate various inositol phospholipids at the D3 position of their inositol head groups. Although PI3Ks are capable of phosphorylating a number of PIs, possibly the most important and certainly the best characterised of these reactions is the 3-phosphorylation of the lipid $PI(4,5)P_2$, giving rise to the second messenger, $PI(3,4,5)P_3$ (Vanhaesebroeck et al., 2001;Wymann and Marone, 2005).

PI3Ks enjoy a relatively ubiquitous expression pattern across phyla, though they are apparently not expressed in plants, and in agreement with this, neither $PI(3,4,)P_2$ nor PIP_3 have been discovered in plant systems (Katso et al., 2001;Vanhaesebroeck et al., 2001). In mammals, the family of PI3Ks has been divided into three major classes, on the basis both of structure and of mechanism of activation. PI3K family members are ordinarily heterodimeric proteins consisting of a catalytic subunit and a regulatory subunit; the various subunit combinations are shown in Figure 1.6.



Class	Subunits		Regulation	Lipid Substrates	
	Catalytic	Adaptor		*In vitro*	*In vivo*
I					
A	p110α,β,δ	p85α,β,γ	Tyr. kinases & Ras	PI, PI(4)P, $PI(4,5)P_2$	$PI(4,5)P_2$
B	p110γ	p101	Het. Tri. G proteins, Ras		
II	PI3K-C2α,β,δ	?	Tyr. Kinases? Het. Tri. G proteins? *Not Ras.*	PI, PI(4)P, [$PI(4,5)P_2$]	?
III	Vps34p analogues	p150	Constitutive?	PI	PI

Figure 1.6
Structural and Regulatory Characteristics of Mammalian PI3Ks. Detailed are the domain structures of mammalian class I/II/III PI3Ks. Key: ○, adaptor-binding domain; ◆, Ras-binding domain; ▬, PI-kinase domain; ⬭, kinase core domain; ▲, C2 domain. Various regulatory pathways of PI3K have been described including tyrosine (Tyr.) kinases and the G-protein Ras, as well as Heterotrimeric (Het. Tri) G-proteins. Various phosphatidylinositol substrate specificities have been described both *in vitro* and *in vivo*. (Adapted from Vanhaesebroeck et al., 1999).

Class I PI3Ks represent the best characterised sub-family of the enzyme, and are themselves further divided into classes I_A and I_B. Three different isoforms of the class I_A catalytic subunit have been characterised: p110α, -β and -δ. These form complexes with one of seven different regulatory or adapter proteins, generated

from the alternative splicing of three genes: p85α and -β and p55γ. Class I_A PI3Ks are activated almost exclusively by signalling events from receptor tyrosine kinases (RTKs), and their regulatory subunits contain a number of SH2 domains, which bind phosphotyrosine (pY)-containing motifs on activated RTKs (Cully et al., 2006). Activation may proceed via a variety of mechanisms; p85 is able to bind directly to pY motifs on activated receptors, itself then directly activating the catalytic activity of the p110 subunit (Domchek et al., 1992). Alternatively, activation by RTKs can involve intermediates such as the adapter Grb2 (Ong et al., 2001), or the large scaffold GAB (Cully et al., 2006). Class I_B consists of the p100γ subunit complexed with the p101 regulatory subunit. This complex is activated either by signalling from the small GTPase Ras, or by signalling from heterotrimeric G-proteins (Cully et al., 2006). Classes II and III have yet to be characterised extensively. Three mammalian class II catalytic subunits exist, although no regulatory subunit has yet been defined (Fig. 1.4.5). Class III PI3Ks are orthologues of the yeast protein Vps34p, which is implicated in vesicular sorting (Vanhaesebroeck and Waterfield, 1999).

1.4.6. The PI3K Signalling Pathway

Possibly the most physiologically significant PI3Ks are the Class I enzymes, which can phosphorylate $PI(4,5)P_2$, to generate $PI(3,4,5)P_3$ (PIP_3), an important second messenger. This reaction takes place in sub-cellular locations rich in anionic phospholipids such as $PI(4,5)P_2$, for example in the inner leaflet of the plasma membrane (Vanhaesebroeck et al., 2001). The so-called PI3K signalling pathway (Figure 1.7) is of great cellular importance, since the generation of PIP_3 allows the recruitment of Plextrin Homology (PH) domain-containing proteins to the plasma membrane. Two particularly important examples of this are the Phosphatidylinositol-dependent Kinase, PDK1, and Protein Kinase B (PKB/Akt) (Cully et al., 2006). Membrane-recruitment of these proteins facilitates the phosphorylation and concomitant activation of PKB/Akt by PDK1. Active PKB/Akt is then able to phosphorylate various other targets, and

in doing so modulates cellular behaviours such as cell growth, proliferation, cell cycle control and transcription (Cully et al., 2006;Wymann and Marone, 2005).

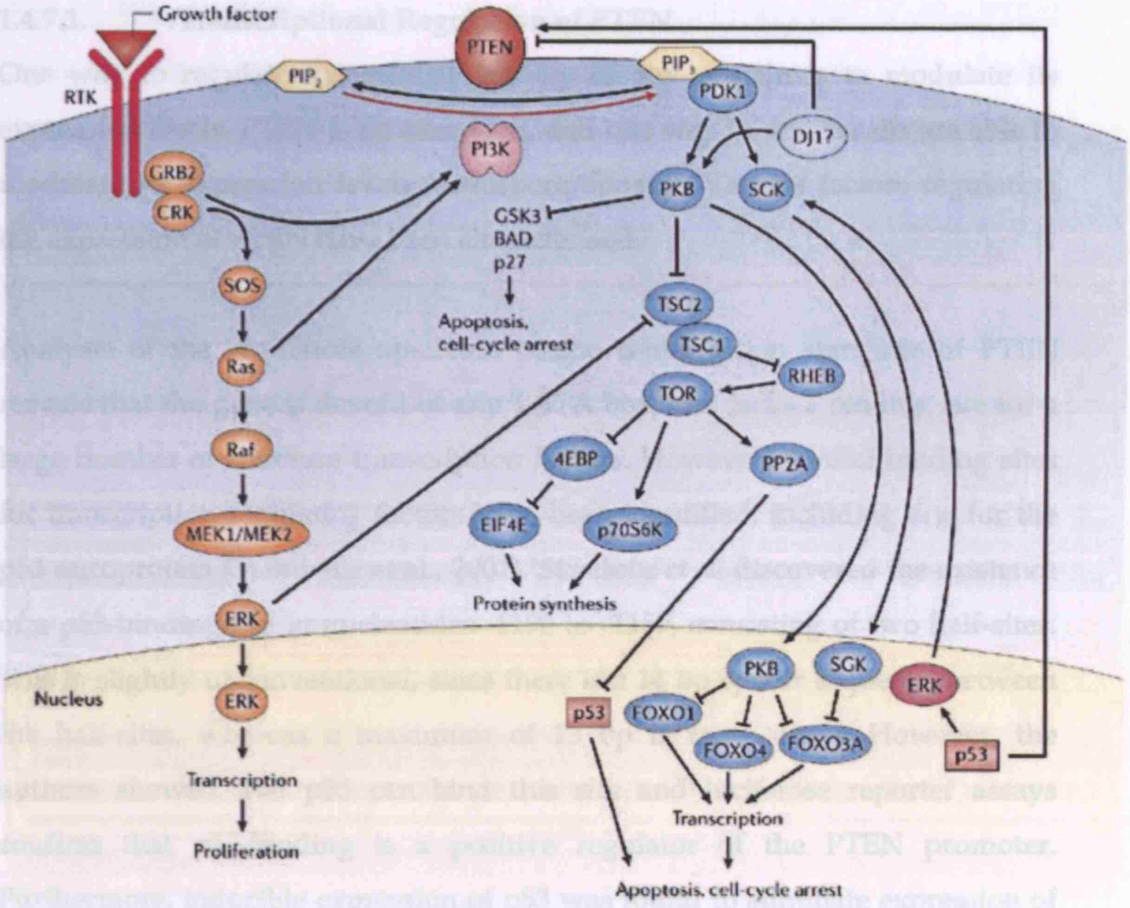


Figure 1.7
The Mammalian PI3K Signalling Pathway. Receptor tyrosine kinases (RTKs) can activate PI3K, leading to PIP_3 synthesis, with concomitant membrane-recruitment of phosphatidylinositol-dependent kinase 1 (PDK1), resulting in phosphorylation and activation of PKB/Akt. There is cross-talk between signalling pathways downstream of RTKs (orange) and signalling pathways downstream of PI3K (blue), leading to highly coordinated regulation of cellular behaviours such as transcription and cell proliferation, as well as apoptosis and cell-cycle arrest. Hydrolysis of PIP_3 by PTEN (red) negatively regulates PI3K signalling (Cully et al., 2006).

1.4.7. Regulation of PTEN

Given the varied and significant range of cellular behaviours controlled by PIP_3, cells must employ a precise mechanism to control the levels of this lipid. Since PTEN has the crucial role of dephosphorylating PIP_3, one aspect of cellular

regulation of PIP_3 levels is centred on the regulation of PTEN activity. The ways in which PTEN is regulated are equally complex and varied.

1.4.7.1. Transcriptional Regulation of PTEN

One way to regulate the cellular activity of any protein is to modulate its expression levels. PTEN is no exception, and one way in which cells are able to modulate its expression levels is transcriptionally. Various factors regulating the expression of PTEN have been characterised.

Analysis of the sequences upstream of the transcription start site of PTEN reveals that the gene is devoid of any TATA box, and lacks a binding site for a large number of common transcription factors. However, several binding sites for transcription-regulating factors have been identified, including one for the p53 oncoprotein (Stambolic et al., 2001). *Stambolic et al.* discovered the existence of a p53-binding site at nucleotides -1190 to -1157, consisting of two half-sites. This is slightly unconventional, since there is a 14 bp spacer sequence between the half-sites, whereas a maximum of 13 bp is more usual. However, the authors showed that p53 can bind this site and luciferase reporter assays confirm that p53-binding is a positive regulator of the PTEN promoter. Furthermore, inducible expression of p53 was found to stimulate expression of PTEN at both the mRNA and protein levels. Consistent with this, the level of phosphorylation of PKB/Akt is greatly diminished in comparison to p53-null cells. Finally, the authors showed that wild-type MEFs underwent apoptosis upon p53 expression, whereas apoptosis did not occur in PTEN-knock out MEFs. This suggests that p53-regulation of PTEN transcription is physiologically relevant, and that PTEN-signalling may represent one important way in which p53 promotes apoptosis.

Another study showed that the early growth response 1 (egr1) transcription factor can also regulate PTEN expression (Virolle et al., 2001). Indeed, mutations in egr2 can give rise to a phenotype similar to that resulting from

PTEN mutation. Several putative egr1-binding sites were found to exist in a region 2kb upstream of the PTEN transcription start site and egr1-expression can stimulate expression of PTEN at the mRNA level. Further, the authors showed using luciferase assays that an increase in activity of the PTEN promoter occurred on stimulation of cells with exogenous egr1, DNA-damaging agents such as etopside, UV-C light or γ-radiation. This did not occur in egr1 knockout cells. These results were confirmed *in vivo* when irradiation of mice wild-type for egr1 showed increased PTEN expression, whereas egr1-null mice did not show this response.

A recent study revealed a method of negatively-regulating PTEN expression by c-Jun, a member of the AP-1 family of transcription factors (Hettinger et al., 2006). C-Jun can induce or inhibit apoptosis, depending on cell type. In the case of cell survival, one interesting possibility is that c-Jun acts by down-regulating the expression of PTEN. C-Jun was found to bind a region in the 5′ upstream sequences of the PTEN gene, and further mapping of the binding site showed it to be a variant AP-1 binding site. Induction of c-Jun activates PKB/Akt, through phosphorylation at Ser473, and luciferase reporter assays showed that this was a consequence of the down-regulation of PTEN expression.

Another way in which the expression of PTEN can be transcriptionally regulated is through methylation of the promoter, which is rich in CpG motifs. PTEN promoter-methylation has been found in many tumour cells such as glioblastomas, endometrial carcinomas, gastric carcinomas and non-small cell lung cancer (Baeza et al., 2003). Modulation of PTEN expression by promoter methylation may therefore be an important epigenetic feature in cancer.

1.4.7.2. Phosphorylation

Another common general mechanism for regulating protein activity is through phosphorylation, and various phosphorylation sites have been identified on PTEN.

Initial experiments showed that neither the pre-phosphatase N-terminal domain nor the post-C2 domain C-terminal regions of PTEN were necessary for functional phosphatase activity (Vazquez et al., 2000). However, a truncated mutant lacking any residues after the C2 domain (PTEN:1-353) was expressed at a fourfold lower level than wild-type PTEN. Interestingly, using the activity of the PKB/Akt pathway as a read out of PTEN activity, the authors showed that PTEN:1-353 was more active than wild-type PTEN, despite its lower expression levels. Taken together, these results suggest that the C-terminal tail of PTEN (residues 353-403) regulates both protein stability and activity. Analysis of data from ^{32}P labelling experiments and thin layer chromatography showed that the tail region is phosphorylated mainly on serine residues, with some threonine phosphorylation, but not on tyrosine residues. Through alanine mutagenesis studies of candidate residues in the tail sequences, the precise phosphorylation sites were mapped to Ser380, Thr382, Thr383 and Ser385. The authors proposed that phosphorylation of the C-terminal tail sequence of PTEN might be responsible for its role in regulating the catalytic activity and protein stability. Consistent with this hypothesis, alanine substitution at the phosphorylated serine and threonine residues gave rise to lower levels of expression than wild-type PTEN, but the mutant protein was more active. Similarly, substitution of a phosphomimetic aspartate residue was sufficient to rescue protein stability, but resulted in a PTEN species whose activity was comparable to that of the wild-type protein, and lower than that of truncated (PTEN:1-353) or alanine mutants.

Another study (Torres and Pulido, 2001) confirmed the phosphorylation-rich nature of the PTEN C-terminus, and suggested that PTEN is constitutively phosphorylated under standard growth conditions. The authors observed that the C-terminal tail sequence contained several putative CK2 (formerly Casein Kinase 2) phosphorylation sites. CK2 is in fact a messenger-independent kinase (Litchfield, 2003), and consistent with this, PTEN purified from bacteria, and

hence un-phosphorylated, could be phosphorylated by purified CK2. Alanine mutagenesis experiments showed that mutation of either Ser370 or Ser385 was sufficient to almost completely abrogate phosphorylation of the PTEN C-terminus, whereas similar mutations at Ser380, Thr382 or Thr383 reduced phosphorylation levels to a lesser degree. However, mutation of Ser370 and Ser385 to aspartate was able to rescue the hyper-phosphorylated state of the C-terminal tail. Finally, the authors carried out experiments with proteasome inhibitors, during which the half-life of either wild-type PTEN or the PTEN quadruple Ala mutant were measured; in both cases treatment with proteasome inhibitors was able to partially rescue PTEN expression levels. Indeed, the stretch of phosphorylated residues in the C-terminal tail is coincident with a putative PEST sequence (Georgescu et al., 1999), implicated in targeting of proteins for proteolytic degradation. Further work later showed that PTEN is cleaved by caspase-3, and that phosphorylation of the C-terminal tail negatively regulates cleavage (Torres et al., 2003). Taken together, these results suggest that CK2 is responsible for phosphorylation of at least two residues (Ser370, Ser 385) in the C-terminal tail sequence, and possible more (Ser380, Thr382, Thr 383). Phosphorylation of Ser370 and Ser385 appears to facilitate further phosphorylation of the C-terminal tail, possibly by priming. Finally, the phosphorylation of this tail sequence of PTEN appears to modulate protein half-life via negative regulation of cleavage of PTEN by caspase-3.

The precise mechanism by which the catalytic activity of PTEN is regulated by phosphorylation of the tail sequence is not well characterised. One possibility is that the phosphorylated tail can wrap around the phosphatase and C2 domains and modulate catalytic activity conformationally (Vazquez et al., 2000), or by preventing membrane association ((Vazquez et al., 2000;Das et al., 2003), see below).

The model suggested by these studies was both refined and complicated by a further study conducted in T-lymphocytes which suggested that S380 is not

phosphorylated by CK2 (Al-Khouri et al., 2005). Immunoblotting of extracts of Jurkat cells treated with RNAi against CK2α/α' (the catalytic subunits) with an α-PTEN:PhosphoSer380 antibody showed no effect on S380 phosphorylation (Vazquez et al., 2000). Furthermore, the authors identified two further phosphorylation sites, Ser362 and Thr366, for glycogen synthase kinase 3β (GSK3β), and showed that CK2-phosphorylation of Ser370 effectively primed the C-terminal tail for further phosphorylation of Ser362 and Thr366 by GSK3β. Although no insights have yet been gained into the role of Ser362 phosphorylation, phosphorylation of Thr366 has been implicated in negatively regulating PTEN activity, and in regulating interaction with the oncogenic protein MSP58 (Okumura et al., 2005).

One particularly interesting study has characterised the regulation of PTEN by Rho GTPases (Li et al., 2005). In mouse neutrophils, RhoA and Cdc42 were able to regulate the intracellular localisation of PTEN. Constitutively active RhoA stimulated the lipid phosphatase activity of PTEN, resulting in a decrease in phosphorylation of PKB/Akt. Interestingly, although Cdc42 alone did not have this effect, it was able to synergize with constitutively active RhoA, to further decrease PKB/Akt phosphorylation. RhoA and PTEN also co-localised at the posterior of migrating cells. The authors showed by Immunoprecipitation experiments that ROCK interacted with and phosphorylated PTEN, and that this phosphorylation was more efficient in the presence of active PI3K. Four potential phosphorylation sites were identified by mass spectroscopy: Ser229, Thr223, Thr319 and Thr321. Phosphorylation by ROCK as a result of expression of constitutively active Rho facilitated translocation of PTEN to the cell membrane. These phosphorylation sites were shown to be of importance in chemotaxis, since when Ser229 and Thr223 or all four sites were mutated to alanine, neutrophils were unable to chemotax in response to SDF-1. This study represents an interesting interaction between PTEN and the Rho GTPases.

Finally, a more recent study has implicated the Microtubule-Associated Serine/Threonine Kinases (MAST kinases) MAST3, MAST205 and SAST in PTEN phosphorylation, although the sites have not yet been mapped, and the significance of this phosphorylation is unclear (Valiente et al., 2005).

1.4.7.3. Regulation of Protein-Protein Interactions

Some studies have suggested that the recruitment of PTEN to the membrane may also proceed via interactions with PDZ-domain containing proteins such as MAGI2 (Downes et al., 2004;Wu et al., 2000b;Wu et al., 2000a). Current models have suggested that this may be important for the metabolism of localised pools of PIP_3. Further studies have also posited that this interaction may be regulated by phosphorylation of the PDZ-binding motif of PTEN (Adey et al., 2000), or of the Thr382/383 residues (Tolkacheva et al., 2001).

1.4.7.4. Sub-cellular Localisation

Since cellular PIP_3 is predominantly, if not exclusively, found in the plasma membrane, PTEN must be recruited here to be functionally active. Although this was previously thought to be mediated by the C2 domain, one study has suggested that this is not energetically feasible (Das et al., 2003). In fact, recruitment to membranes may more likely be effected by the N-terminal PIP_2-binding motif (Walker et al., 2004). In addition to the direct regulatory effect of recruiting PTEN to sites where its substrate is present, membrane-binding may regulate PTEN's catalytic activity through allosteric activation by PIP_2 (Campbell et al., 2003). Finally, there is evidence to suggest that the phosphorylation status of the C-terminal tail of PTEN regulates membrane-binding. Thus PTEN which is hyper-phosphorylated is maintained in a cytosolic localisation, through an electrostatic effect resulting from repulsion between the anionic membrane lipids and phosphate moieties on PTEN (Downes et al., 2004).

1.4.7.5. Reactive Oxygen Species

One particularly interesting mechanism of regulating PTEN activity relates to the redox environment of the cell. Although they were originally considered somewhat deleterious by-products of cellular biochemistry, Reactive Oxygen Species (ROS) are emerging as potentially important regulators of cellular behaviour and signalling (Hool, 2006;Heneberg and Draber, 2005;Salmeen and Barford, 2005;Sauer and Wartenberg, 2005;Zmijewski et al., 2005). Regulation by ROS may be a feature common to all PTPs, by virtue of their active site cysteine residue which is crucial for catalytic activity, and potentially oxidisable. One study in particular has addressed the potential role of ROS in the regulation of PTEN catalytic activity (Leslie et al., 2003). The PTEN active site contains a crucial catalytic residue Cys124, which is positioned close to another cysteine residue, Cys71. The authors argue that redox conditions within the cytoplasm cause both residues to be oxidised, giving rise to the reversible formation of a disulphide bond between them, and thus inhibiting PTEN catalytic activity. A number of lines of experimental evidence support this argument. Firstly, PTEN is active *in vitro* only under reducing conditions. Secondly, in an assay in which cysteine residues are modified by an irreversible alkylating reagent, samples where PTEN was previously oxidised by H_2O_2 were able to have catalytic activity rescued, whereas samples where no such prior oxidation took place were irreversibly inactivated by alkylation. This suggests that oxidation protects the active site cysteine from alkylation, probably by means of disulphide bridge formation. In further support of this model, treatment of cells with H_2O_2 causes an increase in cellular PIP_3 levels, reflected in increased levels of phospho-PKB/Akt. Together, these results suggest that signalling by ROS could represent a significant mechanism of PTEN regulation *in vivo*.

1.4.8. Cellular Roles of PTEN

PTEN was initially characterised as a tumour suppressor. A major way in which PTEN is able to bring about tumour suppression is via transcriptional regulation of other proteins through phosphatase-dependent mechanisms,

which antagonise the PI3K pathway. An example of this is the transcriptional regulation of the mdm2 oncogene. A recent study determined that PTEN is responsible for regulating transcription of the mdm2 gene at its P1 promoter (Chang et al., 2004). Although the mechanistic details have not yet been fully characterised, it is dependent upon the lipid phosphatase activity of PTEN. In addition, transcription of another gene over-expressed in cancers, c-myc, is also regulated by PTEN, which modulated transcriptional activity at the P1 promoter, in a manner also dependent upon lipid phosphatase activity (Ghosh et al., 1999). Although the mechanism of transcriptional regulation of individual proteins by PTEN remains to be precisely characterised, many phosphatase-dependent transcriptional effects likely rely on attenuation of PKB/Akt signalling, which is an important regulator of the forkhead family of transcription factors (Accili and Arden, 2004), whose role in cancer is attributed to their ability to control the expression of genes controlling cellular activities such as apoptosis and the cell cycle (Burgering and Kops, 2002;Dijkers et al., 2000;Kops and Burgering, 1999;Medema et al., 2000).

Evidence is emerging that PTEN is able to modulate transcription of proteins via phosphatase-independent mechanisms. One intriguing study in mouse embryonic fibroblasts (MEFs) examined the ability of PTEN to form a complex with the p53 tumour suppressor via the PTEN C2 domain (Freeman et al., 2003). PTEN was found to modulate the DNA-binding activity of p53 by increasing its transcriptional activity. Similarly, depletion of PTEN in cells by generating PTEN-deficient or knock-out mice led to decreases in the transcriptional activity of p53. Further, it was also found that PTEN controls the stability of p53 at the post-transcriptional level. Cells from mice with decreased levels of PTEN expression also showed decreased p53 protein levels. One explanation of this was that loss of PTEN leads to hyperactivity of the PKB/Akt pathway (Davies et al., 1999), which in turn leads to hyperphosphorylation and thus nuclear localisation of mdm2, which modulates p53 turnover. However, subsequent experiments also showed that the ability of PTEN to stabilise p53 was also

attributable to mechanisms independent of the PTEN-phosphatase activity and of mdm2 (Freeman et al., 2003). Finally, several studies have shown that PTEN is able to regulate expression of various proteins in ways both dependent and independent of PTEN protein- and lipid-phosphatase activities (Shim et al., 2006;Kim et al., 2003).

Another facet of cellular behaviour regulated by PTEN is the cell cycle, which is also of crucial importance to tumour suppression. The cell cycle in mammalian cells is controlled by a family of proteins termed cyclins, which themselves control the activity of a range of serine/threonine kinases known as cyclin-dependent kinases (CDKs). Many studies have demonstrated the importance of regulation of these proteins by PTEN for proper cell cycling. Much of PTEN's effect on the cell cycle is attributable to signalling which prevents cells from going through the G_1-S transition and various mechanisms for this have been proposed. Firstly, PTEN could regulate cyclin D1 (Radu et al., 2003) and therefore G_1-S transition. Experiments showed that re-establishment of PTEN expression in U87MG glioma cells resulted in a decrease in cyclin D1 protein levels, which was dependent on PTEN phosphatase activity. Secondly, expression of PTEN resulted in decreased nuclear localisation of cyclin D, again in a manner dependent on phosphatase activity. Concomitantly, the retinoblastoma (Rb) protein was less phosphorylated at cyclin D1/CDK4-specific sites than in cells in which PTEN was not expressed. A further study showed that PTEN was able to bring about a similar arrest in the G_1 phase of the cell cycle by inhibiting cyclins E and A, and CDK2. This was shown to be attributable to inhibition of PI3K signalling by PTEN, ultimately implicating the CDK-inhibitor $p27^{Kip1}$ (Li and Sun, 1998). PKB/Akt, when activated by PI3K signalling, phosphorylates $p27^{Kip1}$ which is then retained in the cytoplasm, where it is unable to bring about G_1-arrest (Viglietto et al., 2002;Shin et al., 2002). PTEN reverses this inhibition by antagonising the PI3K signalling pathway, resulting in nuclear localisation of $p27^{Kip1}$, and arrest at the G_1 phase of the cell cycle.

Another important area of cellular behaviour regulated by PTEN is cell survival. PTEN-deficient MEFs are much less sensitive to normally apoptotic stimuli such as UV, TNFα and sorbitol than wild-type cells (Stambolic et al., 1998). Re-introduction of PTEN into PTEN-deficient cells rescues sensitivity to such apoptotic stimuli and inhibits cell proliferation (Stambolic et al., 1998;Flynn et al., 2000). The apoptotic signalling of PTEN is attributable to its antagonism of the PI3K signalling pathway. The Bad protein, a substrate of PKB/Akt, possesses pro-apoptotic properties in its non-phosphorylated state, through interactions with other pro-apoptotic proteins such as BclxL and Bcl2 (Datta et al., 1997;del Peso et al., 1997). When phosphorylated by PKB/Akt, Bad is unable to form complexes with the Bcl proteins, and hence leads to cell survival. PTEN-deficiency leads to an inappropriate loss of apoptotic signalling, which is significant for tumourigenesis (Knobbe et al., 2002).

In the amoeboid *Dictyostelium discoideum*, which readily undergoes chemotaxis in response to a wide variety of cues, including cAMP, PTEN signalling has been shown to be of crucial importance for directional sensing. One study in particular showed that application of a chemotactic gradient initiated the recruitment of PH domains specifically to the front of the cell, towards the source of chemoattractant (Iijima and Devreotes, 2002). This indicated a polarised cellular distribution of PIP_3, which is required for the membrane-recruitment of PH domains, and, concomitant with this, PTEN localised predominantly to the rear of the cell. Similarly, these results have been repeated in mammalian leukocytes. Gene disruption of *Dictyostelium* PTEN resulted in cells which were unable to aggregate in response to cAMP, despite there being no down-regulation of the cAMP receptor. These cells also showed more erratic protrusive activity at the cell membrane, and the recruitment of PH domains to the cell membrane no longer occurred in a polarised manner, suggesting that the distribution of PIP_3 had been perturbed.

PTEN has also been implicated in regulation of cell adhesion and spreading, processes of significance to invasion by cancer cells. The role of PTEN in decreasing cell invasiveness through down-regulation of the PI3K signalling pathway has been amply demonstrated; glioma cells, which often lack PTEN, are notoriously invasive. Further, treatment of gliomas *in vitro* with inhibitors of PI3K such as the LY compound led to a reduction in cell invasion (Knobbe et al., 2002). It has been demonstrated that PTEN can dephosphorylate FAK *in vitro* and since phosphorylation of FAK can lead to its removal from focal adhesions, this may represent an important method of regulating focal adhesion dynamics *in vivo* (Carragher and Frame, 2004). PTEN has been shown to regulate invasiveness and cell migration by other mechanisms. Notably, PTEN and PKB/Akt signalling regulate the expression of matrix metalloproteinases (MMPs) (Moon et al., 2004;Zhang et al., 2004;Zhang and Brodt, 2003), which allow tumour cells to degrade ECM, and thus enjoy freer migration. PTEN expression decreases expression of MMPs, and hence invasiveness.

PTEN may additionally regulate invasiveness of tumour cells at the level of migration. A study by *Liliental et al.* demonstrated that fibroblasts from PTEN-knockout mice migrated more quickly, and that reintroduction of PTEN led to migration at rates similar to those of wild-type cells. Increased cell motility was attributed to increased activity of Rac and Cdc42 (Liliental et al., 2000), which in turn was attributed to activation of GEFs by phosphoinositide signalling. Interestingly, other studies have shown that PTEN can cause a decrease in cell motility in glioma cells in a manner independent of lipid phosphatase activity (Maier et al., 1999).

1.4.9. The PTEN C2-domain: Phosphatase-Independent Signalling as an Important Aspect of PTEN Activity

The importance of the lipid and protein phosphatase activities of PTEN in tumour suppression and other cellular processes has been demonstrated in many studies. However, a number of recent articles have suggested a role for

the C2 domain, or at least a phosphatase-independent role, in various cellular processes such as the regulation of gene and protein expression (Kim et al., 2003) and, perhaps most notably, cell migration (Raftopoulou et al., 2004).

A relatively recent study specifically assigned a role to the PTEN C2 domain in the down-regulation of migration in human glioblastoma cell lines (Raftopoulou et al., 2004). An initial characterisation of the motile behaviour of three human glioma cell lines (U373, U138 and U87; U373 cells are the least aggressive followed by U138 and U87, both of which are particularly aggressive) showed that these cells displayed aberrant migration. Both the U87 and U138 cell lines were able to migrate extensively in the absence of serum, and did not show contact inhibition and all three cells lines displayed an increased rate of migration, relative to primary astrocytes (Raftopoulou, 2003). Since each of these cell lines lack any PTEN expression (Raftopoulou, 2003;Knobbe et al., 2002), it was speculated that PIP_3 levels might be unusually high and that this could provoke an increase in the activity of Rho GTPases, particularly Rac, by affecting the activities of GAPs and/or GEFs. In order to test this hypothesis, PTEN was reintroduced into U373 cells in the context of a wound-healing assay. A confluent monolayer of cells was scratched, and after a recovery period, the front line of cells was injected with a myc-tagged PTEN construct, cells were allowed to migrate for 18 hours, and then fixed and stained for myc. As expected, the myc-PTEN expressing cells had been overtaken, and were now far behind the leading edge of the monolayer, indicating that their migration had been inhibited by the introduction of exogenous PTEN. Surprisingly, when the phosphatase and C2 domains were injected separately in the same assay, the anti-migratory property of PTEN was attributable not to the phosphatase domain, but to the C2 domain (Raftopoulou and Hall, 2004;Raftopoulou, 2003). Perhaps even more surprising was the finding that although the PTEN-G129E mutant - which lacks lipid phosphatase activity, but retains this activity towards protein substrates - was able to inhibit cell migration, a mutant lacking all catalytic activity - C124S - had no anti-

migratory effect. It was concluded that although the C2 domain was responsible for the anti-migratory activity of PTEN displayed in the wound-healing assay, this could be regulated by PTEN's protein phosphatase activity in the context of the full-length protein. Simultaneously, the Thr383 phosphorylation site of PTEN was identified as being of considerable importance for the regulation of the C2 domain; specifically, a phosphomimetic mutation of this residue to aspartate (T383D) abolished all anti-migratory activity of the wild-type, full-length PTEN, whereas mutation of the same residue to alanine rescued the ability of PTEN-C124S to inhibit cell migration. Taken together, these results led to the proposal that PTEN might perform an autodephosphorylation at Thr383, which would result in a conformational change, resulting in the activation of the C2 domain's ability to inhibit cell migration. This could be reversed either by degradation of the PTEN protein, or by phosphorylation of Thr383 by an as yet uncharacterised kinase (Fig. 1.8).

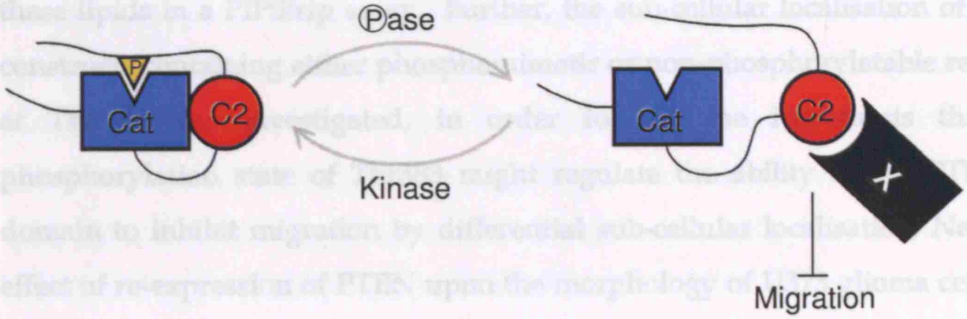


Figure 1.8
Model of Regulation of Anti-migratory Signalling by PTEN C2 domain. Recent data detailing an anti-migratory signalling activity of PTEN's C2 domain resulted in the proposition of a model where dephosphorylation of a regulatory threonine residue in the C-terminal tail (T383) would give rise to a conformational change, rendering the C2 domain more accessible to a proposed interactor molecule, X. C2-X complex formation was proposed to lead to abrogation of cell motility (Raftopoulou et al., 2004).

Although the role of PTEN in down-regulating various aspects of cell migration via its lipid phosphatase activity had been amply demonstrated previously, and

although the ability of PTEN-G129E to inhibit cell migration had also been shown (Maier et al., 1999), the regulation of this latter inhibition by phosphorylation at Thr383 was a novel finding. An attempt to better characterise the mechanism by which PTEN effects its anti-migratory activity is the subject of this thesis. Firstly, an attempt was made to further utilise the wound-healing assay to evaluate the effects of microinjection of PTEN mutant constructs on cell migration of U373 cells. The problems encountered in this work are detailed below, together with a discussion of possible solutions. Secondly, a bioinformatics approach was employed, in order to determine whether the PTEN C2 domain might share homology with a known protein, previously implicated in regulation of cell motility, and thus potentially provide a means of understanding the anti-migratory signalling of the C2 domain. Next, the hypothesis that the C2 domain might inhibit cell migration by sequestration of phospholipid signalling molecules implicated in regulation of motility was tested by assaying the ability of the PTEN C2 domain to bind these lipids in a PIPStrip assay. Further, the sub-cellular localisation of PTEN constructs containing either phosphomimetic or non-phosphorylatable residues at Thr383 was investigated, in order to test the hypothesis that the phosphorylation state of Thr383 might regulate the ability of the PTEN C2 domain to inhibit migration by differential sub-cellular localisation. Next, the effect of re-expression of PTEN upon the morphology of U373 glioma cells was observed, in order to determine whether this caused a morphological defect, and whether this might illuminate an underlying defect in cell signalling, which could account for the anti-migratory effect. Finally, the ability of U373 glioma cells to polarise in response to wounding was assayed, and the effect of expression of PTEN constructs on Golgi polarity was investigated, in order to determine whether the migration-inhibiting effect of PTEN-C2 could be attributed to a defect in correct polarisation.

Chapter 2:

Materials and Methods

2.1. Cell Biology

2.1.1. Cell culture

Cos7 cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM, GibcoBRL), supplemented with 10% Foetal Calf Serum (Mycoplex), and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin; GibcoBRL). Cells were maintained in an incubator at 37°C with 10% CO_2, and were passaged every two days. To passage, cells were rinsed with sterile PBS A, which was then aspirated. In order to release cells from their substrate, 2 ml Trypsin-EDTA (GibcoBRL) were added and cells incubated with for 4 minutes, after which cells were detached by gentle tapping. Eight ml culture medium (see above) were added to inactivate trypsin, and cells were resuspended thoroughly, before adding 2 ml to a fresh flask of medium.

U373 glioma cells (Bertoglio et al., 1987) were maintained in Modified Eagle's Medium (MEM, GibcoBRL) supplemented with 10% Heat-inactivated Foetal Calf Serum (Mycoplex), Non-Essential Amino Acids (GibcoBRL), 10 mM HEPES (GibcoBRL) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin; GibcoBRL). Serum was heat-inactivated by incubating for 45 minutes at 56°C. Cells were cultured in an incubator at 37°C with 5% CO_2, and were passaged every two days, following the procedure for Cos7/HeLa cells. Cells were discarded upon reaching passage 20.

2.1.2. Transfection

Cos7 cells were transfected using GeneJuice (Invitrogen). After passage, cells were seeded into wells of a 6-well plate (Nunc) at a density of 1×10^5 cells/well, and incubated for 24 hours. Three µl GeneJuice were mixed with 100 µl unsupplemented DMEM (GibcoBRL) for each transfection reaction. The mixture was lightly vortexed and incubated at room temperature for 5 minutes, after which time 0.5/1 µg DNA, as required, was added, and this mixture incubated for a further 10 minutes. Finally, the mix was added in a dropwise manner to wells containing Cos7 cells, and cells were returned to the incubator for a further 24 hours before experimentation.

2.1.3. Nucleofection

U373 glioma cells were nucleofected using an Amaxa nucleofection device as follows. After passage, 2×10^6 cells were aliquotted into a centrifuge tube and collected by pelleting in a benchtop centrifuge at 900 rpm for 5 minutes. The resulting pellet was washed thoroughly with sterile PBS A, and cells collected by centrifugation. Cells were resuspended in 100 µl Nucleofector Solution V (Amaxa) and 4 µg DNA of interest were added. This mixture was transferred into a nucleofection cuvette, and air bubbles removed by gently tapping. Cells were nucleofected using programme A23 and immediately after nucleofection, 400 µl warmed culture medium were added, and the mixture plated into a culture dish of appropriate size, containing glass coverslips for later immunofluorescence staining.

2.1.4. Microinjection

After passage, U373 glioma cells were seeded onto glass coverslips in 4-well plates (Nunc) at a density of 7.6×10^4 cells/well, and mixed thoroughly by pipetting. After 48 hours, cells in a wounded, confluent monolayer were injected using an Eppendorf Micromanipulator 5171 and transjector 5240 system on a Zeiss Axiovert 135M microscope in a 5% CO_2 and 37°C controlled chamber. DNA for microinjection was prepared by MaxiPrep (Qiagen, see

below) and diluted into PBS A. In order to remove any debris, diluted DNA solutions were centrifuged through a SpinX Column (Costar) for 30 minutes prior to microinjection. Alexa-594 dextran (Molecular Probes) for co-injection was added at a final concentration of 5 mg/ml.

2.1.5. Wound Healing Assay

The wound healing assay to analyze cell migration or polarity in U373 cells was carried out as follows. Cells were cultured for microinjection as described above. To analyze cell migration, confluent monolayers of cells were scratched using a P100 pipette tip; medium containing any debris was aspirated and replaced with fresh medium. Cells were then incubated at 37°C/5%CO_2 for 30 minutes to recover. Simultaneously, 5 ml glioma culture medium in a 6 cm culture dish were equilibrated at 37°C/5%CO_2, for at least 30 minutes. When microinjection was initiated, coverslips containing scratched monolayers of cells were transferred to the 6 cm dishes of equilibrated medium, and microinjected with DNA constructs over a period of 30 minutes using the facility described. In order to assay their effects on migration of U373 glioma cells, myc-tagged PTEN constructs were injected into cells occupying the row of the monolayer closest to the wound. After 30 minutes injection time, coverslips were returned to the incubator, and cells were allowed to migrate for 18 hours post-scratching, after which time coverslips were fixed and stained with TRITC-phalloidin and 9E10 α-myc antibodies by immunofluorescence, as described below. Injected cells were classified as having been inhibited or not either by determining whether cells had remained in the first row of cells, adjacent to the wound, or by determining the distance of cells from the wound using Metamorph software.

In order to assay cell polarity, scratched, confluent monolayers of U373 cells were prepared as for the wound-healing migration assay detailed above. In order to demonstrate the capacity of U373 cells to polarise relative to a wound, wounded monolayers were simply allowed to recover in the incubator for 2.5, 4 and 7 hours, after which time they were fixed and stained with the Hoechst

nuclear stain, TRITC-phalloidin to visualise actin and antibodies to the p115 Golgi marker (CRUK, Lincoln's Inn Fields). In order to assay the effect of PTEN constructs on Golgi polarisation in U373 cells scratched, confluent monolayers were prepared as previously. Myc-tagged PTEN constructs, or the control empty vector pRK5myc, were then co-injected into the row of cells nearest to the wound together with the injection marker Alexa594 Dextran (Molecular Probes), beginning 30 minutes post-wounding, and continuing for 30 minutes. Cells were then returned to the incubator for 4 hours, after which time coverslips were fixed and stained with an antibody to the p115 Golgi marker by immunofluorescence, as detailed below.

2.1.6. Immunofluorescence

Cells were stained by immunofluorescence as follows. Untransfected cells or those expressing a construct of interest were rinsed once in PBS A at the appropriate time, and fixed for 15 minutes using 4% para-formaldehyde-pH 7.6 in PBS A. Para-formaldehyde was aspirated, cells were rinsed once in PBS A and remaining active para-formaldehyde was removed by incubation of the cells for 15 minutes in 50 mM NH_4Cl (Sigma). Cells were washed once again with PBS A, which was then aspirated, and cells were incubated for 1 hour in blocking solution (10% Goat Serum (Sigma), 0.1% Triton-X100 (Sigma) in PBSA) in order to permeabilise cells and to prevent non-specific binding of antibody. The blocking solution was aspirated, and cells were incubated for 45 minutes with primary antibodies diluted in PBSA as detailed below. After incubation, antibody solutions were aspirated, and coverslips washed gently three times in PBS A to remove residual primary antibody. Cells were incubated with dye-conjugated secondary antibodies (Molecular Probes) for 45 minutes in light-free conditions, diluted in PBS A as detailed below. The antibody solution was aspirated, cells were washed three further times, and finally stained (if appropriate) with either Hoechst (at 5 μg/ml), and/or TRITC-coupled phalloidin at 50 ng/ml for 10 minutes, before rinsing three final times, and mounting onto slides in Dako Fluorescence Mounting Medium (Dako

Cytomation). Prepared slides were allowed to dry overnight before visualisation using a Zeiss Axioskop Microscope. Images were captured with a Jenoptik ProgRes C14 camera, using OpenLab software.

2.1.7. Antibodies used for Immunofluorescence

The following antibodies, diluted in PBS A as detailed below, were used for immunofluorescence staining of samples (Epitopes are all human): 9E10 α-myc (Mouse, Cancer Research UK, Lincoln's Inn Fields, London - for myc-tagged constructs): 1:100; α-p115 (Mouse, Golgi marker, CRUK): 1:100. Secondary antibodies coupled to TexasRed or Alexa488 (Molecular Probes) were used at 1:800.

2.1.8. Calculation of Cell Morphology Parameters

U373 glioma cells were nucleofected with GFP, wtPTEN or PTEN-C2 (all myc-tagged except GFP) as detailed above. After 24 hours, coverslips of nucleofected cells were fixed and stained, and images captured as detailed above. Metamorph software (Molecular Devices) was used to measure cell perimeter (μm), cell surface area (μm^2) and cell elongation ratio. The cell elongation ratio was calculated by dividing the length of the longest line possible in the cell by that of the longest perpendicular line. Data were plotted graphically using Excel (Microsoft).

2.2. Molecular Biology

2.2.1. List of Constructs Used

Name	Identity	Source	Species	Vector
GFP	Green Fluorescent Protein	Clontech	-	pEGFP-C1
RFPF	Farnesylated Red Fluorescent Protein	J. Cau		
RFP-PAK CRIB	CRIB domain of PAK1	J. Cau	-	pmRFP-C1
wtPTEN	Wild-type PTEN	M. Raftopoulou	Human	pRK5myc
PTEN-C124S	Catalytically dead PTEN	M. Raftopoulou	Human	pRK5myc
PTEN-C2	PTEN C2-domain (residues 175-353)	M. Raftopoulou	Human	pRK5myc
PTEN-C2	PTEN C2-domain (residues 175-353)	I. Mannion (subcloned from pRK5myc)	Human	pGEX2T
PTEN-CS/TA	Catalytically dead PTEN non-phosphorylatable at T383 (T383A).	M. Raftopoulou	Human	pRK5myc
PTEN-CS/TD	Catalytically dead PTEN with a phospho-mimetic mutation at T383 (T383D).	M. Raftopoulou	Human	pRK5myc
PTEN-Dcat	Residues 179-403 of PTEN.	M. Raftopoulou	Human	pRK5myc
N17Rac	Dominant negative Rac	A. Self	Human	pRK5myc
Cyt-C2B	C2B domain of synaptotagmin	M. Raftopoulou/ D. Cutler	Human	pRK5myc
Cyt-C2B	C2B domain of synaptotagmin	I. Mannion (subcloned from pRK5myc)	Human	pGEX2T
GST	Glutathione-S-Transferase	Clontech		pGEX2T
Hrs1-FYVE	FYVE domain from Hrs1	M. Lemmon	*S. cerevisiae*	pGEX2T
PLC -PH	PH domain from PLC	M. Lemmon	*S. cerevisiae*	pGEX2T

2.2.2. Restriction Digests, Phosphatase Treatment of Linearised Vector and Purification of DNA fragments

Restriction digests were carried out in a 50 µl volume by incubating 5 µg of the construct of interest with 10U of each restriction enzyme with 5 µl of the appropriate 10× restriction enzyme buffer (all NEBiolabs) for 2.5 hours at 37°C. For cloning, linearised DNA was treated with calf intestinal phosphatase (CIP) by adding 13U of CIP (NEBiolabs), followed by incubation for 15 minutes at

37°C. CIP was deactivated by freezing on dry ice, followed by incubating for 20 minutes at 75°C. Restriction digests carried out to generate insert for sub-cloning reactions were not subjected to phosphatase-treatment. DNA fragments were purified by electrophoresis of digested fragments on a 1% agarose, made with TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA). DNA bands were visualised using a hand-held UV-lamp, and excised. DNA was purified from excised bands using the QIAquick kit (Qiagen), according to the manufacturer's instructions, with the following modifications: (1) isopropanol was not added after the gel slice was dissolved (Step 5); (2) 0.5 ml of Buffer QG was not added to the QIAquick column (Step 9). DNA was eluted finally in 50 µl sterile, distilled, DNAse/RNAse-free water.

2.2.3. Ligations

Ligations were carried out by incubating 3 µl insert DNA and 1µl vector DNA (purified as above) with 0.5 µl 10× ligase buffer and 0.5 µl (2.5U) T4-DNA ligase (Roche) for 1 hour at room temperature, and then transformed into competent *E. coli*, as detailed below.

2.2.4. Preparation of Competent *E. coli*

Bacteria of the relevant strain (DH5α for DNA production, BL-21 for protein biochemistry) were streaked from a glycerol stock onto an agar plate containing the appropriate antibiotics (50 µg/ml ampicillin for DH5α, with additionally 25 µg/ml chloramphenicol for BL-21). These were incubated overnight at 37°C. A single colony was picked from the plate, and used to inoculate 50 ml L-broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, in 1 litre water) in a sterile Erlenmeyer flask overnight in an orbital shaker at 37°C. A 10 ml overnight culture was used to further inoculate 200 ml L-broth, and this was then shaken at 37°C until the OD_{550} measurement reached 0.6. The bacteria were then incubated on ice for 5 minutes, before transferring to a cold 250 ml conical centrifuge tube and centrifuging at 4000 rpm, 4°C for 15 minutes. The pellet was

then resuspended in 100 ml cold 0.1 M $CaCl_2$ (Sigma), and incubated on ice for 30 minutes. The bacteria were re-centrifuged for 10 minutes at 4,000 rpm at 4°C. The resulting pellet was resuspended in 10 ml 15% glycerol, 50 mM $CaCl_2$, aliquotted, snap-frozen in liquid nitrogen, and stored at -80°C until needed.

2.2.5. Transformation of Competent *E. coli*

50 µl competent *E. coli* of relevant strain (prepared as above) were incubated with 0.5-1 µg plasmid DNA for 30 minutes on ice. Bacteria were heat-shocked by incubating for 2 minutes at 37°C, before incubating on ice for a further 5 minutes. 500 µl sterile, antibiotic-free L-broth was added to the bacterial suspension, which was incubated at 37°C for 30 minutes, after which time the bacteria were centrifuged in an Eppendorf bench centrifuge for 5 minutes at full speed, and resuspended in 100 µl L-broth. This was spread onto an agar plate, containing the appropriate antibiotic to select for the transformed plasmid, and incubated overnight.

2.2.6. Preparation of DNA from *E. coli* DH5α cultures

Plasmid DNA of interest was transformed into DH5α bacteria as described above. A single colony was picked from a plate containing transformants, and used to inoculate 200 ml of L-broth containing the relevant antibiotic. This was incubated overnight at 37°C in an orbital shaker. Bacteria were pelleted by centrifugation for 15 minutes in a Beckman J6-MC centrifuge. The pellet was resuspended in 10 ml Buffer P1 (Qiagen) and DNA was extracted using the MaxiPrep kit (Qiagen) according to the manufacturer's instructions. DNA was eluted into TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA), and OD_{260}/OD_{280} measured in order to calculate DNA concentration and assay quality of resulting DNA.

2.3. Protein Biochemistry

2.3.1. Protein Purification from *E. coli* BL-21 Bacteria

DNA encoding a protein of interest was subcloned into the pGEX2T expression vector (if necessary) for production of glutathione-S-transferase (GST) fusion proteins. A 100 ml overnight culture of plasmid-containing *E. coli* BL-21 cells was incubated at 37°C with shaking and used to inoculate 1 litre of L-broth supplemented with ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml). This culture was incubated at 37°C with shaking for 2 hours, after which time it was transferred to an orbital shaker at 22°C, and allowed to cool for 3 hours. Isopropylthiogalactoside (IPTG) was added to a final a concentration of 0.1 mM to induce protein expression overnight and cells were collected by centrifugation at 4,000 rpm for 10 minutes at 4°C in a Beckman J6MC centrifuge, The pellet was resuspended in 20 ml resuspension solution (20 mM Tris-pH8.0), and the suspension re-centrifuged. The resulting supernatant was discarded and the pellet was snap frozen in liquid nitrogen, before being resuspended in 10 ml lysis buffer (20 mM Tris-pH8.0, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 complete protease inhibitor tablet (Roche)). DNA was sheared by sonication on ice in 10 second bursts. The lysate was centrifuged at 10,000 rpm for 50 minutes at 4°C. The supernatant was removed carefully into a 50 ml Falcon tube, and 1 ml of a 50% slurry of glutathione-agarose beads was added, and the mixture rotated at 4°C for 2 hours. Glutathione beads were harvested by centrifugation at 2,500 rpm in a benchtop centrifuge at 4°C for 30 seconds. Supernatant was removed, and the beads were washed five times with 10 ml cold buffer A (50 mM Tris pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$), collecting the beads each time by centrifugation at 4°C for 30 seconds at 2,500 rpm. Finally, fusion proteins were eluted from the beads by incubating twice for 30 minutes with 500 μl glutathione elution buffer (50 mM Tris-pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM glutathione). Proteins were concentrated by centrifugation in a centricon device (Amicon) until the volume was reduced to approximately 150 μl. Protein concentration was assayed by BCA Assay (Pierce) according to

the manufacturers instructions, and purity was assayed by running samples on a 10% polyacrylamide electrophoresis gel and staining with Coomassie brilliant blue stain, as detailed below (section 2.3.3).

2.3.2. PIP Strip Assay to Determine Phosphoinositide Binding

In order to assay the phosphoinositide binding specificity of the C2 domain of PTEN, the C2B domain of synaptotagmin, the PH domains of PLCδ and Dyn1 and the FYVE domain from Hrs1, a PIP Strip assay was used as follows. GST-fusion protein of these C2 domains were either obtained from other sources, or created by subcloning DNA encoding them into the pGEX2T vector (see sections 2.2.2-2.2.3) and purifying fusion protein as detailed in section 2.3.1.

The PIP Strip (Echelon Biosciences) membrane was first blocked in Tris-Buffered Saline with 0.1% Tween (Sigma; TBST: 25 mM Tris-HCl, pH 8.0; 125 mM NaCl; 0.1% Tween 20), supplemented with 3% fatty acid-free Bovine Serum Albumin (FA-Free BSA, Sigma), and agitated gently at room temperature for one hour. The membrane was incubated overnight with gentle agitation with the fusion protein of interest at 0.5 μg/ml in TBST/3% FA-Free BSA (TBST/BSA). After washing three times for 10 minutes each time with TBST/BSA, the membrane was incubated with a goat-anti-GST antibody (Amersham) at a dilution of 1:1,000 in TBST/BSA for one hour, with gentle agitation at room temperature. The membrane was washed three times and incubated with a Horseradish Peroxidase (HRP)-coupled mouse anti-goat IgG secondary antibody (Pierce), diluted 1:5,000 in TBST/BSA for one hour, again at room temperature with gentle agitation. Finally, after washing three times HRP signal was detected using Enhanced Chemiluminescence (ECL - Amersham) reagents, according to the manufacturer's instructions.

2.3.3. Sodium-Dodecylsulphate Poly-acrylamide Gel Electrophoresis

Protein expression in cell lysates was assayed as follows. Cell lysates were boiled in 5x sample buffer (250mM Tris-HCl pH 6.8, 0.5M DTT, 10% SDS, 50% glycerol, 0.1% bromophenol blue), then loaded into wells of a polyacrylamide gel (stacking gel: 5% acrylamide/ bisacrylamide solution, 125mM TrisHCl pH6.8, 0.1% SDS; resolving gel: 10 - 15 % acrylamide/ bisacrylamide solution as required, 375mM TrisHCl pH8.8, 0.1% SDS), along with 10 µl Molecular Weight Markers (Invitrogen/Amersham). Proteins were resolved by electrophoresis at 90-120V using a Biorad Msinigel apparatus. Proteins in the gel were detected by staining with Coomassie Brilliant Blue solution (0.5g brilliant blue R-250, 10% acetic acid, 50% methanol) for 3 hours, followed by destaining overnight in destain solution [10% acetic acid (v/v) / 25% methanol (v/v)], such that bands were visible. Gels were imaged using standard scanning equipment.

2.4. Bioinformatics

Protein sequences were retrived from the Pubmed facility of the NCBI website (www.ncbi.nlm.nih.gov). Protein-protein BLAST searches were carried out using the BLAST facility of the same website. Protein sequence alignments were carried out using the ClustalX application (Thompson et al., 1997), and alignment figures were prepared using MacBoxShade software, which is in the public domain. 3D protein structure images were produced by visualising .pdb files downloaded from the Brookhaven database (www.rcsb.org/pdb) using CHIME (www.life.uiuc.edu/crofts/bioph2/pdb/chime_talk/download.html).

Chapter 3:

Results

3.1. Summary

Gliomas are malignant cells arising from astrocytes, the principal macroglial cells of the central nervous system. They are characterised by a marked increase in motility when compared to the parent astrocyte lineage from which they derive, and are often highly invasive (Raftopoulou et al., 2004;Raftopoulou, 2003). The PTEN protein was initially identified in a screen for a potential tumour suppressor on chromosome 10, and the phosphatidylinositol phosphatase activity of PTEN is crucial to its tumour suppressor activity. PTEN, through its effects of PIP_3 levels, acts in numerous signalling pathways to control a wide variety of cellular behaviours, including cell migration (Knobbe et al., 2002). However, recent experiments have shown that the isolated C-terminal C2 domain of PTEN is able to negatively regulate the migration of cells independently of any phosphatase activity (Raftopoulou et al., 2004). In this thesis, the mechanism of this activity of the PTEN C2 domain is investigated. Firstly, the wound-healing assay of cell migration is evaluated. Then, a bioinformatics approach is used to identify proteins or domains related in sequence or structure to this domain. Next, the ability of the PTEN C2 domain to bind various classes of phospholipids is examined. Subsequently, the effects of mutations in the C-terminal phosphorylation sites of PTEN on its sub-cellular localisation are examined. Finally, the effects of PTEN expression on cell morphology and polarity investigated.

3.2. The Wound-healing Assay for Cell Migration

A recent study by *Raftopoulou et al.* demonstrated the important result that the re-introduction of PTEN into U373 glioma cells, which do not express PTEN, resulted in an inhibition of migration in these cells in a manner which was unexpectedly due to an activity of the C2 domain rather than the phosphatase domain (Raftopoulou et al., 2004). Central to this work was the wound-healing assay of cell migration, which permitted the re-introduction of PTEN expression into cells occupying the row of cells in a confluent monolayer immediately adjacent to a wound via microinjection of plasmid DNA. An important tenet of this assay was that analysis of the final position of microinjected cells should reveal the nature of the effect of the DNA construct injected; a construct inhibiting migration would be expected to cause injected cells to be overtaken by other cells and thus be found behind the leading edge of the wound at the end of the assay, whilst in the case where a non-inhibitory construct were injected, it would be expected that cells would be found in a position immediately adjacent to the wound. Thus, microinjection of control plasmids, such as GFP or the empty vector pRK5myc, did not inhibit migration and so led to cells being found in the row adjacent to the wound at 18 hours post-scratching, whilst microinjection of wild-type PTEN or a construct encoding the PTEN C2 domain alone resulted in cells being found in rows further back from the leading edge of the wound (Raftopoulou et al., 2004;Raftopoulou, 2003).

Given the centrality of this assay to the study by *Raftopoulou et al.*, it was attempted to repeat the results, in order to validate this assay as a means of investigating the effect of a wider range of PTEN mutant constructs on U373 cell migration. Confluent monolayers of U373 glioma cells were grown on 13 mm glass coverslips and wounded with a P100 pipette tip, according to the protocol established by *Raftopoulou et al.* 30 minutes post-wounding, cells in the row adjacent to the wound were then microinjected with control vectors (such as GFP) or myc-tagged PTEN constructs (as used by *Raftopoulou*) for 30 minutes,

after which time they were returned to the incubator and allowed to migrate for 18 hours. After this time, they were fixed and stained by immunofluorescence to reveal the position of injected cells. Initial analysis of this assay used the criteria detailed by *Raftopoulou*, namely that only cells in the row immediately adjacent to the wound at the conclusion of the assay were classed as displaying normal migratory behaviour, and all other cells were classed as having been inhibited in migration (Raftopoulou, 2003).

Construct	Assay 1	Assay 2	Assay 3	Assay 4	Mean	Raftopoulou, 2003
GFP	47	52			49.5±3.5	75±2.5
RFPF	47				--	--
wtPTEN	43	65	44	27	44.75±15.6	20±1
PTEN-C2	16	30			23±9.9	23±15
PTEN-C124S	71	24	56	52	50.75±19.6	67±7.6

Table 3.1
Results of Wound-healing Assay of Cell Migration in U373 Cells
U373 cells were cultured on glass coverslips and grown to confluence, scratched and microinjected with control (GFP/RFPF) or PTEN constructs. Values denote percentages of injected cells in wound-healing assays remaining in the row of cells immediately adjacent to the wound edge ('Mean' denotes mean percentages, ± standard deviation), in comparison with the mean values (± standard deviation) obtained by *Raftopoulou*, 2003. Results obtained differed significantly from those of *Raftopoulou* ($p = 2.19\times10^{-9}$; χ^2 test, 3 degrees of freedom). GFP: Green Fluorescent Protein; RFPF: Farnesylated Red Fluorescent Protein; wtPTEN: Wild-type PTEN.

An extensive number of wound-healing assays were conducted, a representative sample of which is displayed in Table 3.1, in conjunction with mean results obtained by *Raftopoulou* (Raftopoulou, 2003). As can be observed, there are often wide divergences not only between the results obtained here and those of *Raftopoulou*, but also within the results obtained (Table 3.1). GFP was chosen as a control construct since it has previously been shown not to hinder the ability of U373 cells to migrate in the context of this assay (Raftopoulou et al., 2004;Raftopoulou, 2003) and since it allows microinjected cells to be

visualised with ease, without the need for further immunofluorescence. As shown in Table 3.1, whilst repeated assays demonstrated that a mean value of 49.5%±3.5 of cells microinjected with GFP were found adjacent to the wound, this was consistently lower than the value (75%±2.5) obtained by Raftopoulou. In order to evaluate the efficacy of GFP as a control, cells were microinjected with the control vector RFPF (Farnesylated Red Fluorescent Protein), a vector commonly used as a negative control in the wound-healing assay of cell polarisation (Cau and Hall, 2005). Again, only 47% of cells were found immediately adjacent to the wound (Table 3.1). This suggests a lower efficacy of the wound-healing assay than experienced by *Raftopoulou et al.*; the reason for this is not clear, since the cells and conditions used were identical, but the similar result achieved in cells microinjected with RFPF suggests that the discrepancy observed is not due to GFP. The proportion of cells microinjected with a plasmid encoding PTEN-C2 present at the leading edge of the wound was much lower than for GFP (Table 3.1), perhaps confirming the ability of this domain to inhibit cell migration. One particularly problematic feature of the results obtained for other constructs was the wide variation observed between replicates of other plasmids microinjected, for example wild-type PTEN and PTEN-C124S (Table 3.1). In addition to being significantly divergent from the values reported by *Raftopoulou* for these constructs (Raftopoulou, 2003) ($p = 2.19\times10^{-9}$; χ^2=43.24, 3 degrees of freedom), the variability within the results obtained here for individual constructs (as evidenced by the standard deviation) was consistently greater than that of *Raftopoulou*, suggesting that a comparison between the results obtained here and other results would not be possible. In addition, the proportions of U373 glioma cells injected with wild-type PTEN, PTEN-C2 or PTEN-C124S found adjacent to the wound were not found to differ significantly from GFP control values (p values of a heteroskadistic Student's t test for constructs relative to GFP: wild-type PTEN: 0.60; PTEN-C2: 0.13; PTEN-C124S: 0.91). Given these results, it was concluded that the wound-healing assay of cell migration, with the criteria of

inhibition of migration used here, could not be reliably employed as a means of investigating the effect of novel PTEN constructs on migration in this project.

An attempt was made to adapt the assay detailed above to allow the classification of cell migration in an objective manner. In this adapted assay, cells were prepared, wounded, microinjected, allowed to migrate, fixed and stained as before, but the position of cells at the conclusion of the assay was instead analysed by measuring the distance between microinjected cells, and the wound edge using Metamorph software. RFPF was used as a negative control, and the mean distance from the wound edge of cells microinjected with each of wild-type PTEN, PTEN-C2, PTENΔCAT (res. 179-403) and PTEN-C124S was measured. Since Rac has been demonstrated to be necessary for membrane protrusion in REFs (Cau and Hall, 2005), the dominant-negative species N17Rac was used as a positive control for inhibition of migration. The results of this experiment are detailed in Figure 3.1. As can be observed (Fig. 3.1(a)), the results obtained in these experiments are somewhat at variance with those obtained by *Raftopoulou* (Raftopoulou et al., 2004;Raftopoulou, 2003). Most strikingly, the mean distance from the wound edge of U373 cells injected with the negative control RFPF was 140 μm (~7 cells, where mean cell diameter = 20.3 μm), whereas cells injected with wild-type PTEN were found on average 70 μm (~ 3.5 cells) from the wound. This is a significant discrepancy, and entirely contradicts the results which would be expected on the basis of previous studies (Raftopoulou et al., 2004;Raftopoulou, 2003). Furthermore, cells injected with the PTEN C2 domain or the PTEN C-terminus (PTENΔCat - inhibitory for migration in studies by *Raftopoulou*) or PTEN-C124S were all found at approximately 150 μm (~7.5 cells) from the wound edge. Finally, cells injected with N17Rac, a potent inhibitor of membrane protrusion, were positioned at approximately 200 μm from the wound. In addition to the divergence between the results obtained by *Raftopoulou* (Raftopoulou, 2003) and those obtained here, plotting of a histogram showing frequency distribution of distances from the wound edge of U373 cells injected with, for example, wild-type PTEN

demonstrated that the results obtained remained subject to wide internal variation (Fig. 3.1(b)). Injected cells were distributed at distances between 21-40 μm (1-2 cells, at a mean cell diameter of 20.3 μm) and 161-200 μm (8-10 cells) from the wound

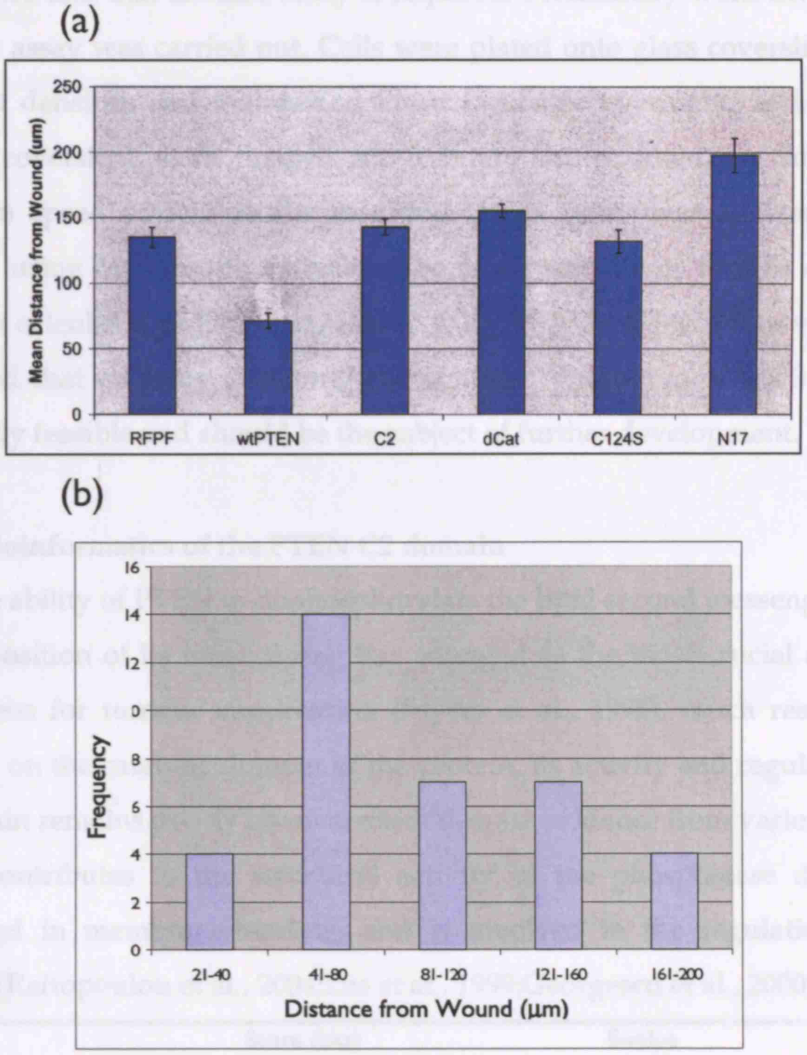


Figure 3.1

The Wound-healing Assay of Cell Migration. (a) U373 cells were grown to confluence on glass coverslips. Monolayers were scratched with a P100 pipette tip, cell culture medium was changed and then cells were returned to the incubator for 30 minutes. After this time, cells were microinjected for 30 minutes with various plasmids encoding either control vectors, or myc-tagged PTEN constructs. Cells were returned to the incubator after injection, and allowed to migrate for 18 hours, after which time they were fixed and stained to visualise injected cells. Distances of individual cells from the wound edge were measured using Metamorph software. Error bars show the Standard Error of the Mean (SEM). RFPF: Farnesylated Red Fluorescent Protein; wtPTEN: wild-type PTEN; C2: PTEN C2 domain; dCat: PTENΔCAT (res. 179-403); C124S: PTEN-C124S; N17: N17-Rac. With the exception of RFPF, all constructs possessed an N-terminal myc-tag. Mean cell diameter was measured using Metamorph software as 20.3 μm. (b) Frequency distribution of distance from the wound edge of U373 cells injected with wild-type PTEN in wound-healing assay and analysed as described for (a) above.

edge. Taken together, these results suggest that the wound-healing assay could not be employed as a means of drawing reliable conclusions regarding the ability of PTEN (or other) constructs to inhibit cell migration in the context of this project, and that another assay is required. Preliminary work investigating one such assay was carried out. Cells were plated onto glass coverslips at sub-confluent densities and maintained in an incubator overnight. 24 hours after plating, coverslips were imaged for 8 hours using time-lapse microscopy. Migration speed of cells in the resulting image series was analysed by cell tracking, using Metamorph software. The mean velocity of 10 cells over the 8 hour was calculated as 0.329 μm/min (± 0.017, 95% confidence interval). It was concluded that an assay observing sub-confluent migration of U373 cells was potentially feasible and should be the subject of further development.

3.3. Bioinformatics of the PTEN C2 domain

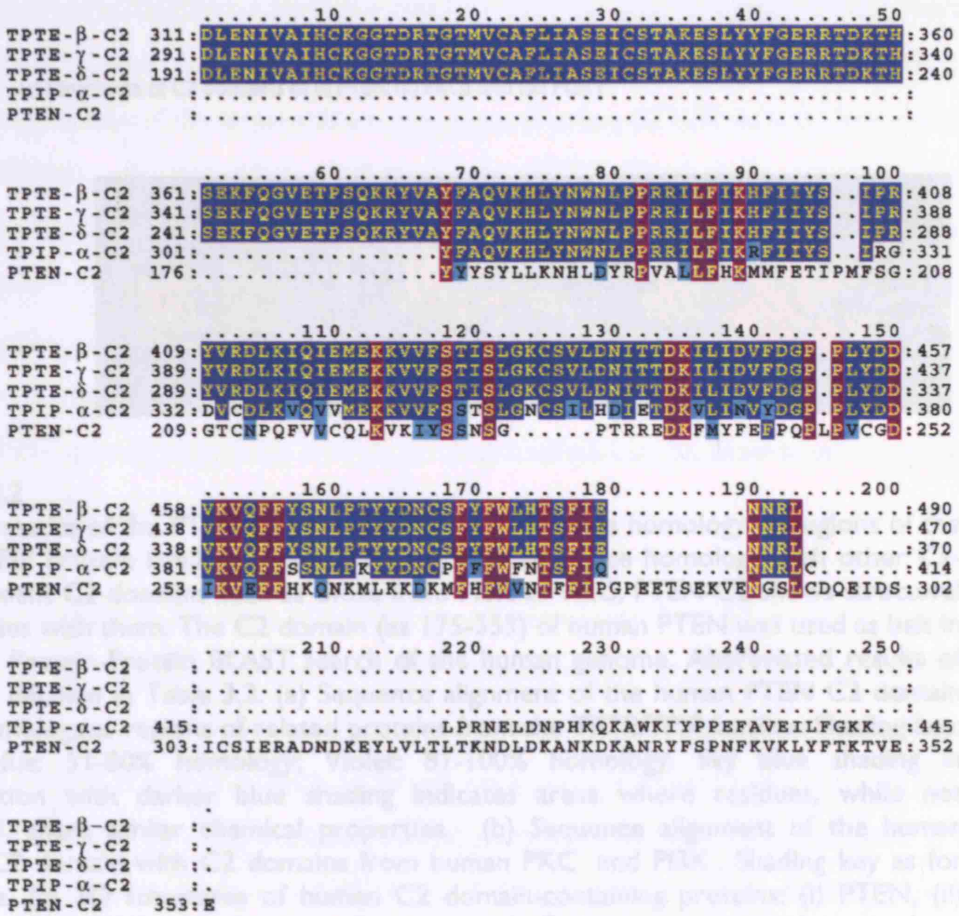
Since the ability of PTEN to dephosphorylate the lipid second messenger PIP_3 at the D3 position of its inositol ring has emerged as the most crucial activity of the protein for tumour suppression (Myers et al., 1998), much research has focussed on the catalytic domain of the protein, its activity and regulation. The C2 domain remains poorly characterised, despite evidence from various studies that it contributes to the structural activity of the phosphatase domain, is implicated in membrane-binding, and is involved in the regulation of cell motility (Raftopoulou et al., 2004;Lee et al., 1999;Georgescu et al., 2000).

Protein	Score (bits)	E-value
PTEN	403	~0
PTEN2	398	~0
Tensin3	37	0.03
C1-TEN	32.3	0.88
TPTE	30.8	2.6
TPTE	30.8	2.6
TPTE	30.4	3.0
TPTE	30.4	3.6

Table 3.2: Results (abbreviated) of an NCBI Blast search against the human PTEN C2 domain.

Figure 3.2

(a) C2 domains from PTEN family members:



(b) C2 domains from other proteins:

```
                  .....10.....20.....30.....40.....50
PTEN-C2      176:....YYYSYLKNHIDYRPVALLFHKMMFETIPMFSGGTCNPQFVVCQLK:221
PKCδ-C2        1:.....MAPFLRIAFNS..........YELGSLQAEDEANQPFCAVKMK:33
PI3Kγ-C2     351:TVSLWDCDRKFRVKIRG.........IDIPVLFRNTDLRVFVEANIQHGQ:383

                  .....60.....70.....80.....90.....100
PTEN-C2      222:VKIYSSNSGPTRREDKFMYFEFPQP......LPVCGDIKVEFFHKQNKML:265
PKCδ-C2       34:EALSIERGKTLVQKKPTMYPEWKS.............TFDAHLYEGR....:66
PI3Kγ-C2     384:QVLCQRRTSPKPFTEEVLWNVWLEFSIKIKDLPKGALLNLQIYCGK....:437

                  .....110.....120.....130.....140.....150
PTEN-C2      266:KKDKMFHFWVNTFFIPGPEETSEKVENGSLCDQEIDSICSIERADNDKEY:315
PKCδ-C2       67:.......VIQIVLMRAAEEPVSEVTVG......VSVLAERCKKNNGKAE:103
PI3Kγ-C2     438:........APALSSKASAESPSSESKGKVQLLYYVNLLLIDHKFLLRRGE:479

                  .....160.....170.....180.....190.
PTEN-C2      316:LVLTLTKND...LEKANKDKANRYFSPNFKVKLYFTKTVEE:353
PKCδ-C2      104:FWLDLQP.....QAKVLMSVQYPLEDVDCKQS.......:130
PI3Kγ-C2 C2  480:VYLHMWQISGKGEDQGSFNADKLTSATNPDKENSMSISILL:520
```

(c) Structures of C2 domains of (i) PTEN, (ii) PKCδ and (iii) PI3Kγ:

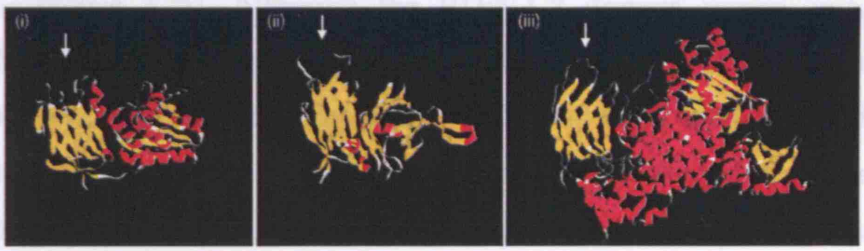


Figure 3.2

Bioinformatics of the PTEN C2 domain. PTEN-C2 displays homology to regions of the TPTE/TPIP protein families. Despite sharing little sequence homology with other Ca-independent C2 domains such as those from PI3K or PKC, PTEN-C2 shows structural similarities with them. The C2 domain (aa 175-353) of human PTEN was used as bait in a NCBI Protein-Protein BLAST search of the human genome. Abbreviated results of this are detailed in Table 3.2. (a) Sequence alignment of the human PTEN C2 domain with homologous regions of related proteins from the TPTE/TPIP families. Shading key: Dark blue: 51-80% homology; Violet: 81-100% homology. Sky blue shading in conjunction with darker blue shading indicates areas where residues, while not identical, share similar chemical properties. (b) Sequence alignment of the human PTEN C2 domain with C2 domains from human PKC and PI3K . Shading key as for Fig. 3.2a. (c) 3D structures of human C2 domain-containing proteins: (i) PTEN, (ii) PKC and (iii) PI3K . α-helices are shown in pink, β-sheets in orange. Arrows show position of C2 domains. Protein sequences were obtained and BLAST searches carried out using the NCBI online facility; alignments were generated using ClustalX and MacBoxShade. 3D protein structures were obtained from the Brockhaven PDB Databank, and images visualised and prepared using CHIME. TPIP: TPTE and PTEN-homologous Inositol lipid Phosphatase; TPTE: Transmembrane Phosphatase with TEnsin homology; PKC: Protein Kinase C; PI3K: Phosphatidylinositol-3-kinase.

An NCBI BLASTp search of the human genome was carried out to identify human proteins with significant sequence homology to the PTEN C2 domain (residues 176-353). The rationale behind this approach was to determine whether PTEN-C2 might show any significant homology to another protein domain, whose signalling capacity might be better understood; in this way, it could be possible to speculate that PTEN-C2 could be involved in similar signalling pathways, and thus begin to elucidate the mechanism of PTEN-C2's anti-migratory signalling. The BLAST results corresponding to known proteins are shown in Table 3.2. Aside from a PTEN pseudogene (PTEN2), tensins2/3 and the tensin homologous phosphatase C1-TEN, the only known proteins with which the PTEN shares a significant homology are TPTE-β, -γ and –δ, and TPIP-α. The sequences of these proteins were aligned using CLUSTALX ((Thompson et al., 1997) - Figure 3.2a). Although the PTEN-C2 domain varies quite considerably from homologous regions in the TPTE/TPIP proteins, there was much less variation between the homologous region in TPTE members and TPIP-α. Nevertheless, some 26 residues were conserved between all domains aligned. Nothing is currently known concerning the cellular function of the TPTE/TPIP family members, and so no conclusions can be drawn, concerning the possible functional implications of this homology.

The C2 domain of PTEN is classified as a Ca-independent C2 domain since, unlike the C2 domains of classical PKC homologues, it does not bind Ca^{2+}-ligands. In order to check the validity of making claims concerning protein or domain structure and/or function based on primary sequence homology, the sequence of the PTEN C2 domain was aligned with the Ca^{-2+} independent C2 domains of protein kinase C-δ (PKCδ) and PI3Kγ (Figure 3.2b). Each of the three domains has been shown to be active in membrane and lipid-binding (Murray and Honig, 2002). Sequence alignment showed that despite this common function, the primary sequences of these domains are very divergent. Further, the 3D structures of these domains, as determined by X-ray crystallography, were obtained from the Brookhaven PDB database, and visualised using Chime

(Figure 3.2c). As can be seen, despite the low level of primary sequence homology, the secondary and tertiary structure of each of the C2 domains is remarkably similar, with all three domains consisting of an anti-parallel β-sheet (arrows). These data suggest that primary sequence homology is not a useful means of drawing conclusions regarding tertiary structure and function of C2 domains.

3.4. Interaction of inositol phospholipids with the PTEN C2 domain

In addition to the structural similarities between the PTEN C2 domain and the Ca^{2+}-independent C2 domains from PKCδ and PI3Kγ, the PTEN C2 domain has been shown to be important for membrane binding by the PTEN protein (Lee et al., 1999;Georgescu et al., 2000), although no study has yet addressed the precise lipid-binding profile of the PTEN C2 domain. Indeed, the prototypical C2 domain of PKC isoforms has been shown to play an important role in lipid-/membrane-binding (Nalefski and Newton, 2001). As a result, a PIPStrip assay was used in order to determine the lipid-binding specificity of the PTEN C2 domain, and to test the hypothesis that PTEN-C2 might bind phospholipid signalling molecules, and potentially exert its anti-migratory signalling by sequestering these molecules when over-expressed in glioma cells. The C2 domain of human PTEN was subcloned into a Glutathione-S-Transferase (GST) bacterial expression vector, in order to generate a GST-fusion protein which could then be purified from *E. coli* extracts. Similarly, GST itself was used as a negative control and the Synaptotagmin (Syt) C2B domain, known to have no effect upon migration of U373 cells in a wound-healing assay (Raftopoulou et al., 2004), was subcloned, expressed and purified as for PTEN-C2, and used as an additional control in the assay.

Vectors encoding GST-PTEN-C2 and GST-Syt-C2B were transformed into BL-21 *E. coli* cells. A colony was picked for each fusion, and grown in LB medium over-night either in standard conditions (Fig. 3.3 (a) and (b) lane 'i') or induced to produce GST-fusions protein by incubating with 0.1 mM IPTG (Fig. 3.3 (a)

and (b) lane 'ii'). Cells incubated with/without IPTG were then harvested by centrifugation, solubilised in sample buffer and samples run on a 10% polyacrylamide gel to check for successful induction of recombinant protein (Figure. 3.3 (a) and (b)). In both cases, overnight induction with IPTG resulted in the presence of a band of the correct molecular weight (GST-C2: ~49 kDa; GST-C2B: ~36 kDa) in induced but not un-induced cells (Fig. 3.3 (a) and (b), arrows), suggesting that these fusion proteins are indeed correctly synthesised by *E. coli* BL-21 cells with no significant degradation and that induction with IPTG is efficient. *E. coli* cells transformed with the relevant plasmid were then used to establish larger cultures, which were induced overnight with 0.1 mM IPTG as before. Recombinant protein was harvested from cells as detailed (section 2.3.1 above). In order to check protein purity and to estimate protein concentration, various volumes of purified protein were then separated on polyacrylamide gels, adjacent to standard quantities of Bovine Serum Albumin (Fig. 3.3 (c) and (d)). Gels were then stained with Coomassie Brilliant Blue, and destained as standard. The resultant staining showed that both fusion proteins purified efficiently. There was negligible contamination of fusion proteins with co-purifying species; additional bands were feint, and appeared only when relatively high quantities of purified protein were loaded. Bands corresponding to each fusion protein also occurred at the expected molecular weight (GST-C2: 49 kDa, GST-C2B: 36 kDa).

Following their successful purification, GST-PTEN-C2 and GST-Syt-C2B were used in PIPStrip assays, in order to determine their phospholipid-binding specificities. GST (kind gift of Bernike Kalverda) was used as a negative control. PIPStrips (Echelon Biosciences), commercial nitrocellulose membranes on which aliquots of phospholipids had been spotted, were blocked in a fatty acid-free BSA solution, before being incubated with solutions of the relevant fusion proteins. After washing the blots, sites of protein binding were visualised by further incubation with anti-GST antibodies, followed by anti-IgG-coupled HRP secondary antibodies, and finally ECL detection as standard. GST-PTEN-C2

interacted specifically with PI(3)P, PI(4)P, PI(5)P, $PI(3,4)P_2$, $PI(3,5)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$, but not PI, or other lipids such as phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), sphingosine-1-phosphate (S1P) or phosphatidic acid (PA) (Figure 3.3f). When PIPStrips were incubated with GST alone, no interactions could be observed, demonstrating that the fusion to GST does not adversely affect the specificity of lipid binding by the C2 domain and that GST does not itself bind any of the lipids present (Figure 3.3e).

Raftopoulou et al. showed that the C2 domain of PTEN inhibited migration of U373 glioma cells *in vitro*, but that the Ca^{2+}-independent C2B domain of a synaptotagmin family member did not (Raftopoulou et al., 2004). This showed that the Ca^{2+}- independent SytC2B exhibited an identical pattern of lipid-binding to that of PTEN-C2 (Figure 3.3g). Since SytC2B is not able to exert an anti-migratory effect in U373 glioma cells, it is possible to conclude that sequestration of these lipids by the PTEN C2 domain is not responsible for the anti-migratory effect reported by *Raftopoulou et al.*

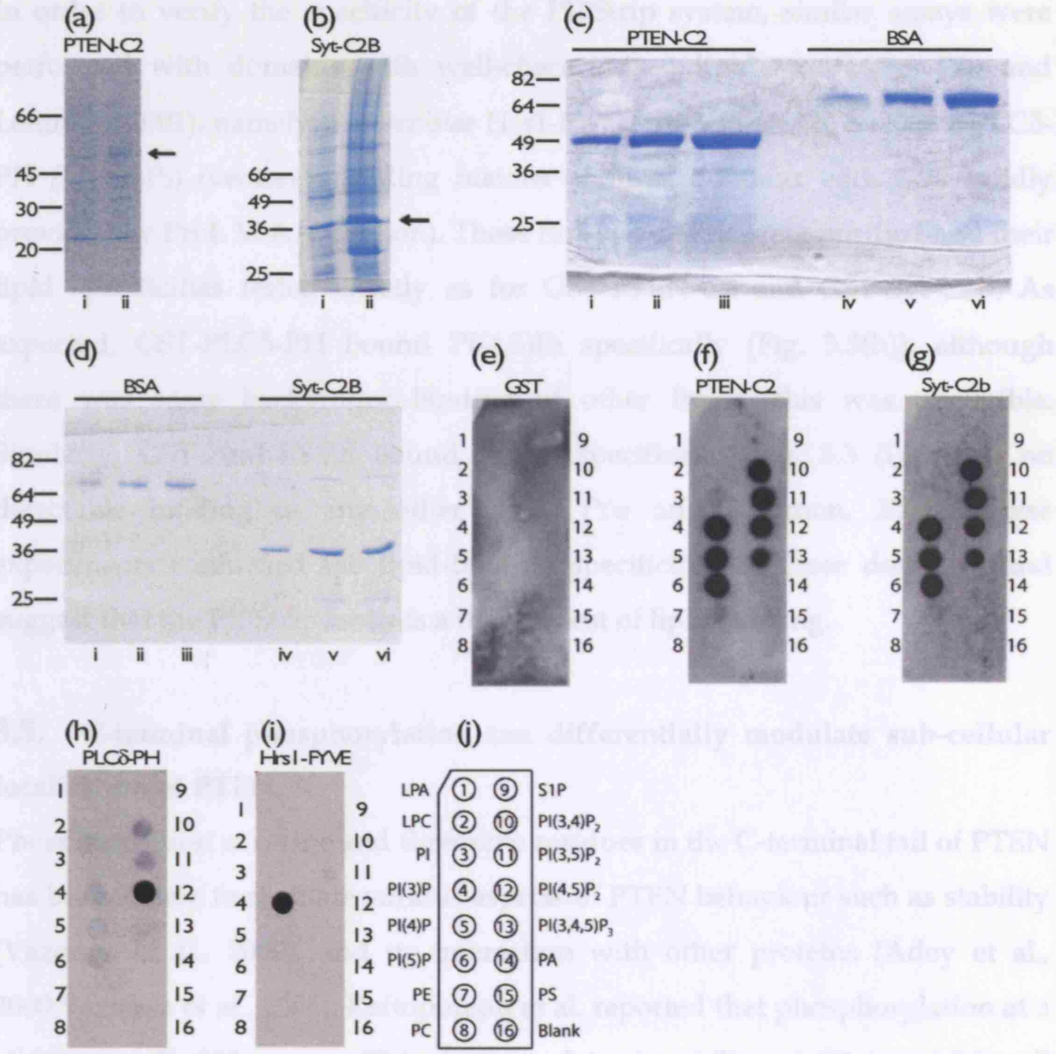


Figure 3.3

PTEN-C2 and Synaptotagmin-C2B bind an identical range of phosphatidylinositols.

The phospholipid binding specificity of the human PTEN C2 domain and other domains was tested by means of a phospholipid PIPStrip. **(a)-(b):** SDS-PAGE of extracts of *E. coli* transformed with plasmids encoding N-terminal fusions of GST with (a) human PTEN C2 and (b) human synaptotagmin (Syt) C2B. In each case, 'i' denotes uninduced *E. coli* cells and 'ii' denotes cells induced overnight with IPTG. **(c):** SDSPAGE of purified human PTEN-C2 and BSA standards. Lanes consist of (i) 2.5 µl (ii) 5 µl and (iii) 10 µl of purified PTEN-C2 and (iv) 2.5 µg (v) 5 µg and (vi) 10 µg of Bovine Serum Albumin (BSA). (d) SDSPAGE of purified human Syt-C2B. Lanes consist of: (i) 1 µl, (ii) 2 µl and (iii) 3 µl of BSA and (iv) 1 µg, (v) 2 µg and (vi) 3 µg of purified Syt-C2B. **(e)-(j)** PIPStrips showing lipid-binding specificity of (e) GST alone and fusions of GST with: (f) human PTEN C2, (g) human synaptotagmin C2B, (h) *S. cerevisiae* PLCδ-PH, (i) *S. cerevisiae* Hrs1-FYVE. (j) Layout of phospholipids on PIPStrip. Fusion proteins were used at 0.5 µg/ml. SDSPAGE: Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis; GST: Glutathione-S-Transferase; BSA: Bovine Serum Albumin; PLC: phospholipase C.

In order to verify the specificity of the PIPStrip system, similar assays were performed with domains with well-characterised lipid-specificities (Yu and Lemmon, 2001), namely: *S. cerevisiae* Hrs1-FYVE (PI-3-P) and *S. cerevisiae* PLCδ-PH ($PI(4,5)P_2$) (vectors encoding fusions of these domains with GST kindly provided by Prof. M.A. Lemmon). These fusion proteins were purified and their lipid specificities tested exactly as for GST-PTEN-C2 and GST-Syt-C2B. As expected, GST-PLCδ-PH bound $PI(4,5)P_2$ specifically (Fig. 3.3(h)); although there was some background-binding of other lipids, this was negligible. Similarly, GST-Hrs1-FYVE bound PI-3-P specifically (Fig. 3.3 (i)), with no detectable binding to any other lipids (Yu and Lemmon, 2001). These experiments confirmed the lipid-binding specificities of these domains, and suggest that the PIPStrip assay is a reliable test of lipid-binding.

3.5. C-terminal phosphorylation can differentially modulate sub-cellular localisation of PTEN.

Phosphorylation at serine and threonine residues in the C-terminal tail of PTEN has been shown to regulate various aspects of PTEN behaviour such as stability (Vazquez et al., 2000), and its interaction with other proteins (Adey et al., 2000;Vazquez et al., 2001). Raftopoulou et al. reported that phosphorylation at a single site, Thr383, was sufficient to regulate the ability of C2 to inhibit cell migration (Raftopoulou et al., 2004). One possibility is that phosphorylation at Thr383 could regulate C2-mediated inhibition of migration by causing a change in sub-cellular localisation, removing the PTEN protein from the environ of some interactor molecule. In order to test this hypothesis, the effect of modulating the phosphorylation state of Thr383 on PTEN behaviour was investigated by transfecting Cos7 cells with either a GFP control (Figure 3.4a), or myc-tagged PTEN constructs as follows: wtPTEN (Fig 3.4b), the catalytically dead PTEN:C124S (Fig 3.4c-d), PTEN-C2 (Fig 3.4e), the non-phosphorylatable PTEN:C124S-T383A (Fig. 3.4f) or the phosphomimetic PTEN:C124S-T383D (Fig. 3.4g). The phosphomimetic/non-phosphorylatable mutants were used in a

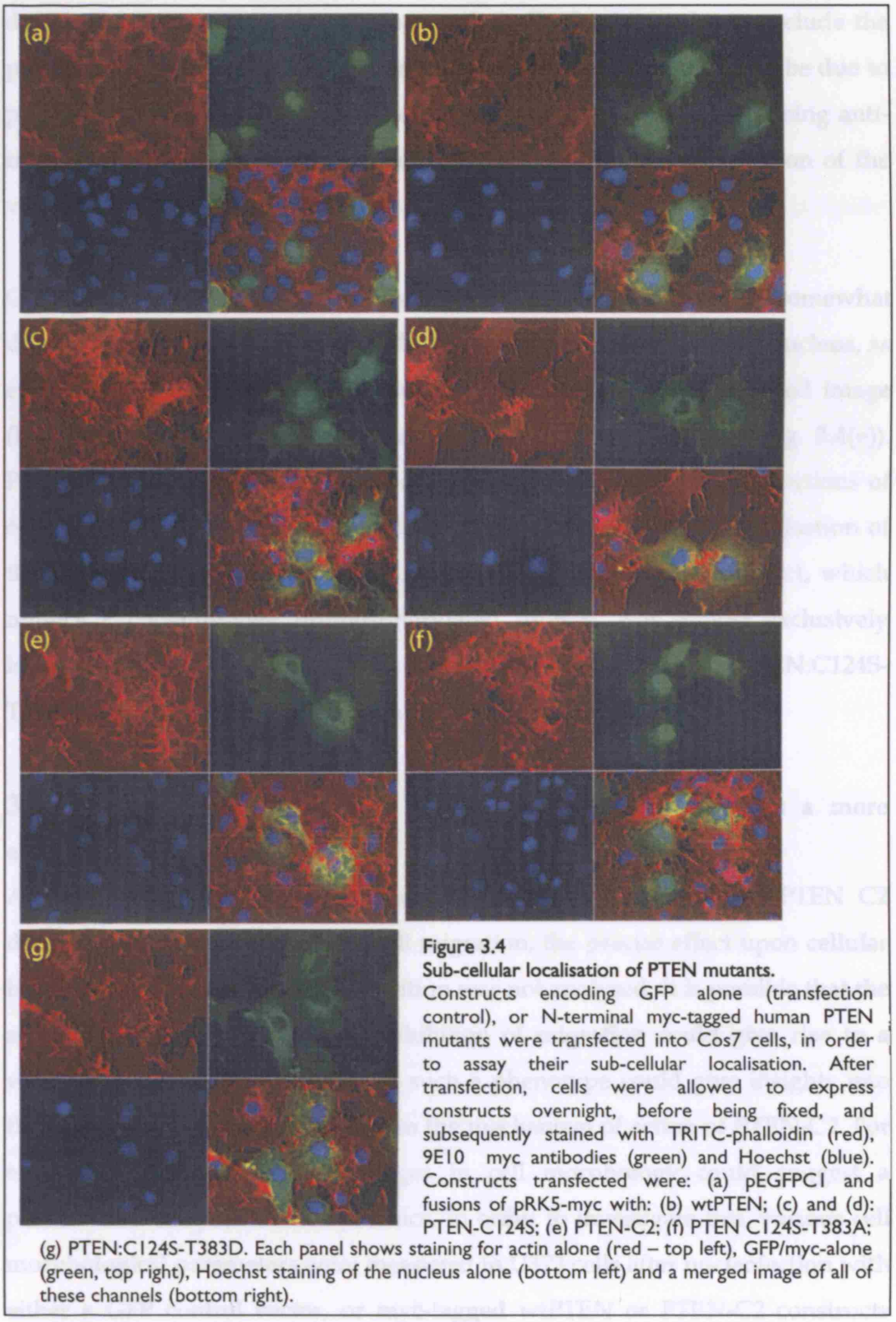


Figure 3.4
Sub-cellular localisation of PTEN mutants.
Constructs encoding GFP alone (transfection control), or N-terminal myc-tagged human PTEN mutants were transfected into Cos7 cells, in order to assay their sub-cellular localisation. After transfection, cells were allowed to express constructs overnight, before being fixed, and subsequently stained with TRITC-phalloidin (red), 9E10 myc antibodies (green) and Hoechst (blue). Constructs transfected were: (a) pEGFPC1 and fusions of pRK5-myc with: (b) wtPTEN; (c) and (d): PTEN-C124S; (e) PTEN-C2; (f) PTEN C124S-T383A; (g) PTEN:C124S-T383D. Each panel shows staining for actin alone (red – top left), GFP/myc-alone (green, top right), Hoechst staining of the nucleus alone (bottom left) and a merged image of all of these channels (bottom right).

double mutant form which was also catalytically dead in order to exclude the possibility that any observed change in sub-cellular localisation could be due to phosphatase activity. Transfected cells were then fixed and stained using anti-myc antibodies and TRITC-phalloidin, and the sub-cellular localisation of the various PTEN constructs observed.

GFP showed a nuclear localisation. Wild-type PTEN showed a somewhat diffuse localisation, which appeared to be largely excluded from the nucleus, as evidenced by the visibility of nuclear Hoechst staining in the merged image (Fig. 3.4(b), bottom right). This was also the case for PTEN-C2 (Fig. 3.4(e)). PTEN-C124S localisation was varied, with approximately equal proportions of cells displaying a nuclear or cytoplasmic (i.e. nuclear excluded) localisation of this construct (Fig. 3.4 (c) and (d)). The PTEN-C124S-T383A construct, which mimics a constitutively unphosphorylated protein, was almost exclusively localised to the nucleus (Fig. 3.4 (f)), whereas the phosphomimetic PTEN:C124S-T383D construct was excluded from the nucleus (Fig. 3.4 (g)).

3.6. Expression of PTEN and PTEN-C2 causes cells to adopt a more elongated morphology.

Although *Raftopoulou et al.* reported that expression the isolated PTEN C2 domain was sufficient to inhibit cell migration, the precise effect upon cellular behaviour responsible for this inhibition was not analysed. It is possible that the signalling event responsible for inhibition of migration could give rise to a visible phenotype. Investigation of such a phenotype could give insights into the underlying cause, and hence into the mechanism of action of PTEN-C2. For example, PTEN-C2-induced changes in cell morphology could suggest a perturbation of cytoskeletal dynamics. In order to investigate this, various cell morphological parameters were measured in U373 cells after nucleofection with either a GFP control vector, or myc-tagged wtPTEN or PTEN-C2 constructs (Figure 3.5a-d). Cells were fixed after 24 hours, stained with TRITC-phalloidin, and with anti-myc antibodies in the case of PTEN or PTEN-C2 expressing cells.

Cells were then imaged, and images analysed using Metamorph analysis software in order to measure cell surface area, cell perimeter and the ratio of longest lengths – a measure of cell elongation where the length of the longest line traceable inside the cell was divided by the length of the longest possible perpendicular line. In addition, the surface area:perimeter ratio was calculated.

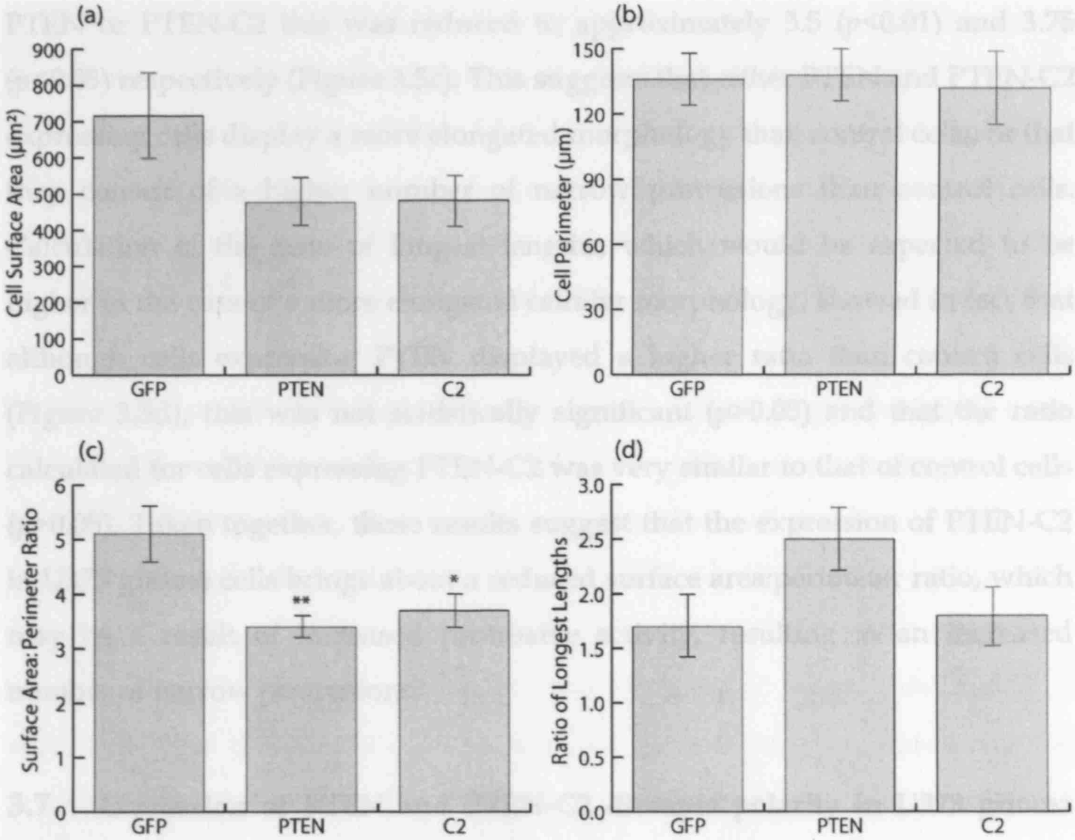


Figure 3.5
PTEN and PTEN-C2 Decrease the Surface Area:Perimeter Ratio of U373 Cells.
U373 cells were nucleofected with a GFP control vector, or with N-terminal myc-tagged PTEN or PTEN-C2 constructs in order to assay the effects of these constructs on U373 morphology. Cells were nucleofected, and fixed and subsequently stained with 9E10 α-myc antibodies 24 hours post nucleofection. Images were obtained as standard, and analysed using Metamorph software. Parameters measured were: (a) cell surface area (μm²); (b) cell perimeter (μm); (c) surface area:perimeter ratio; (d) ratio of longest lengths. Statistical significance was tested using a *t*-test (two-sample, heteroskadistic) in Microsoft Excel. *: $p < 0.05$; **: $p < 0.01$

Analysis of cell surface area (Figure 3.5a) showed that whilst GFP-expressing control cells had a mean surface area of some 700 μm², cells expressing PTEN or

PTEN-C2 had a reduced mean surface area of approximately 475 μm^2, although this difference was not quite statistically significant ($p>0.05$). Conversely, the perimeter of cells expressing GFP, PTEN or PTEN-C2 was remarkably constant, with a mean value of approximately 135 μm^2 in each case (Figure 3.5b). Computation of the surface area:perimeter ratio of these cells showed that whilst GFP-expressing control cells displayed a ratio of 5, in cells expressing PTEN or PTEN-C2 this was reduced to approximately 3.5 ($p<0.01$) and 3.75 ($p<0.05$) respectively (Figure 3.5c). This suggests that either PTEN and PTEN-C2 expressing cells display a more elongated morphology than control cells, or that they consist of a higher number of narrow protrusions than control cells. Calculation of the ratio of longest lengths, which would be expected to be higher in the case of a more elongated cellular morphology, showed in fact that although cells expressing PTEN displayed a higher ratio than control cells (Figure 3.5d), this was not statistically significant ($p>0.05$) and that the ratio calculated for cells expressing PTEN-C2 was very similar to that of control cells ($p>0.05$). Taken together, these results suggest that the expression of PTEN-C2 in U373 glioma cells brings about a reduced surface area:perimeter ratio, which may be a result of increased protrusive activity, resulting in an increased number of narrow protrusions.

3.7. Expression of PTEN and PTEN-C2 disrupts polarity in U373 glioma cells

Correct cellular polarisation in response to external stimuli is a crucial step in properly-directed cell motility, and disruption of cellular polarity is one possible cause of inhibition of cellular migration (Ridley et al., 2003;Cau and Hall, 2005;Etienne-Manneville and Hall, 1999;Hall, 2005). The ability of U373 glioma cells to polarise in this manner has not been documented.

One common method of assaying the ability of cells to polarise *in vitro* is the wound-healing assay (Nobes and Hall, 1999), in which cells are grown to confluence, before a scratch is made in the monolayer using a micropipette tip.

Cells along the front edge of this wound are then microinjected with constructs, whose effect on cellular polarity can be measured when cells are fixed and stained for a polarity marker. One common marker of cell polarity is the position of the microtubule organising centre (MTOC) in the cell, which is ordinarily positioned in front of the nucleus, oriented towards the wound and is indicative of correctly polarised microtubule dynamics (Cau and Hall, 2005;Nobes and Hall, 1999). This is thought to be concomitant with retrograde translocation of the nucleus (Gomes et al., 2005). Another marker for cell polarity is the positioning of the Golgi apparatus, which in properly polarised cells is also ordinarily positioned in front of the nucleus, oriented towards the wound. The position of the Golgi apparatus is thought to accurately reflect the position of the MTOC (Nobes and Hall, 1999).

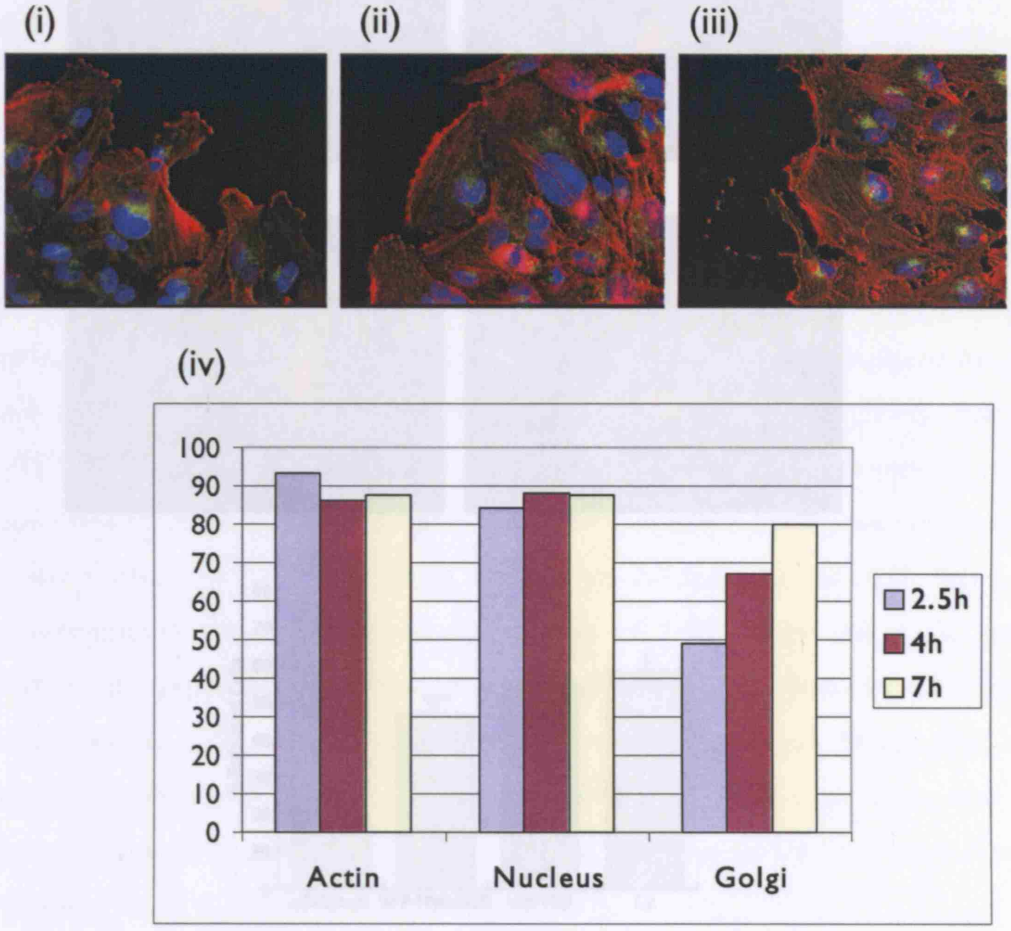
In order to test the ability of U373 cells to polarise, the wound-healing assay (Cau and Hall, 2005) was employed. Cells were grown to confluency on glass coverslips, and confluent monolayers were scratched, using a micropipette tip. Cells were then fixed after 2.5 hours (Fig. 3.6(a)(i)), 4 hours (Fig. 3.6 (a)(ii)) and 7 hours (Fig. 3.6.(a)(iii)), and stained for actin (TRITC-phalloidin, red), the nucleus (Hoechst, blue) and the Golgi apparatus (α-p115, green). The ability of cells to initiate membrane ruffling into the wound area, to translocate the nucleus to the rear of the cell, furthest from the wound edge, and to correctly orient the Golgi apparatus in the 120° sector in front of the nucleus (Cau and Hall, 2005) was quantified (Fig. 3.6 (a)(iv)). It was observed that the ability of cells to correctly initiate membrane ruffling into the wound space was rapid, since even at 2.5 hours post scratching, most (93.5%) cells showed extensive membrane protrusion activity into the wound, most often in the form of broad lamellipodium-like structures (Fig. 3.6 (a)(i), (iv)). Again, considerable protrusive activity in the wound space was also noted in cells at 4 and 7 hours post-scratching, with 86% and 87.5% of these cells showing this at these respective time points (Fig. 3.6 (a)(ii)-(iv)). Unfortunately, the nature of the monolayer prevented the analysis of the proportion of cells displaying back-

protrusions, since it was impossible to differentiate sufficiently between contributions made to actin staining by adjacent cells, hence protrusion polarity could not be assayed. The translocation of the nucleus to the rear half of the cell also occurred rapidly in a large proportion of wound-edge cells, with 84.2%, 88.3% and 87.5% of cells showing a correctly-translocated nucleus at 2.5, 4 and 7 hours respectively (Fig. 3.6 (a)(i)-(iv)). Reorientation of the Golgi apparatus to the 120° sector in front of the nucleus was slower, with 49.1%, 67.2% and 80.0% of cells demonstrating correctly localised Golgi apparatus at 2.5, 4 and 7 hours respectively. Although Golgi re-orientation was slower than the other parameters measured here, both the timescale and threshold level of re-orientation were comparable to similar values as measured in other cell systems such as rat astrocytes (Etienne-Manneville and Hall, 1999) and rat embryonic fibroblasts (Cau and Hall, 2005). Overall, these preliminary experiments demonstrate that U373 glioma cells are able, according to widely-used criteria, to polarise in response to external stimuli such as wounding, and, as a result, to initiate directed migration.

One hypothesis which could explain increased protrusive activity and/or inefficient or inhibited migration in U373 cells expressing PTEN or PTEN-C2 is that these cells could be unable to respond to their environment in a polarised way. In order to test the effect of expression of PTEN constructs on the polarity of U373 glioma cells, and thus whether a polarity defect might be responsible for inhibition of migration by PTEN-C2, a further wound healing assay was conducted, in which cells in a confluent monolayer at the front edge of a wound were microinjected either with an empty vector (pRK5myc), Red Fluorescent Protein-linked PAK:CRIB - a domain known to bind both Rac and Cdc42, and used routinely in the context of such polarity assays (Nobes and Hall, 1999;Etienne-Manneville and Hall, 1999;Cau and Hall, 2005) to inhibit Cdc42 signalling - wild type PTEN, or PTEN-C2. The isolated phosphatase of PTEN was not used in this experiment, since the aim was rather to characterise whether any polarity-perturbing effect of wild-type PTEN might be attributable

to the isolated C2 domain, and hence be potentially related to the anti-migratory effect characterised by *Raftopoulou et al.* (Raftopoulou et al., 2004). Constructs were co-injected with Alexa-594-coupled dextran in order to easily visualise injected cells (Figure 3.6b-e). Cells were then fixed and stained 4 hours post-injection with antibodies to p115, a well-characterised Golgi marker (Cau and Hall, 2005) in order to visualise the localisation of the Golgi apparatus in injected cells. Cells were imaged, and the number of cells where the Golgi apparatus was localised to the 120° sector in front of the nucleus was counted (Figure 3.6f). In samples where cells had been injected with the empty vector pRK5myc, some 75% of cells displayed correct orientation of the Golgi apparatus, showing both that U373 glioma cells are capable of correctly reorienting the Golgi apparatus, and that the technique of microinjection does not adversely affect this polarisation event. Injection of cells with RFP-PAK:CRIB, commonly used to inhibit of Cdc42 in this assay (Cau and Hall, 2005), reduced the proportion of cells with correctly positioned Golgi to approximately 45% ($p<0.001$). Furthermore, cells injected with either wtPTEN or PTEN-C2 displayed a similarly reduced degree of Golgi polarisation, with only 55% ($p<0.001$) and 57.5% ($p<0.05$) of cells respectively having correctly polarised their Golgi apparatus. Since the position of the Golgi apparatus is thought to additionally represent that of the MTOC (Nobes and Hall, 1999) these results, taken together, suggest that PTEN is able to regulate cellular polarity - polarity of the Golgi and potentially MTOC in particular - and that this effect is attributable to the isolated C2 domain.

Figure 3.6 (a)


(i)
(ii)
(iii)
(iv)
100
90
80
70
60
50
40
30
20
10
0
Actin
Nucleus
Golgi
2.5h
4h
7h

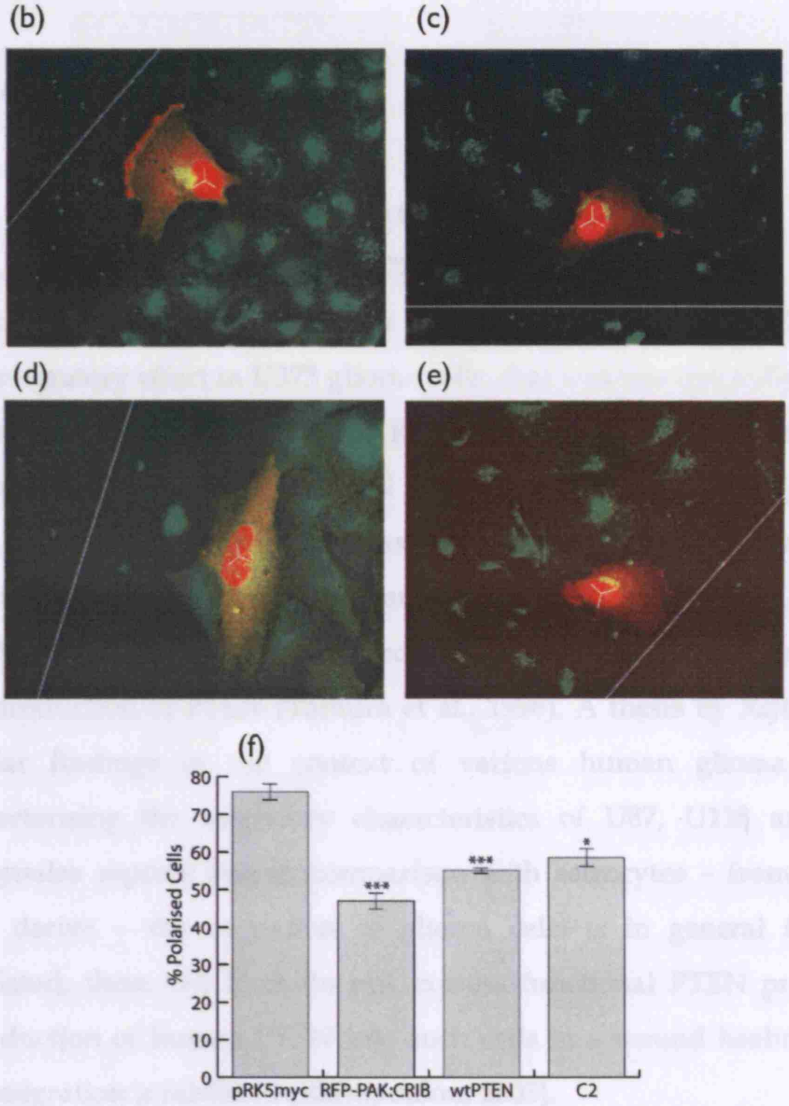


Figure 3.6
Expression of PTEN/PTEN-C2 disrupts Golgi Polarity in U373 cells.
Polarisation in U373 glioma cells was observed using a wound-healing assay. Cells were grown to confluency on a glass coverslip, and a wound was generated by scratching with a micropipette tip. Coverslips containing wounded monolayers were returned to the incubator and fixed at 2.5 hours, 4 hours and 7 hours post-wounding and subsequently stained with TRITC-phalloidin (red), Hoechst (blue) and with antibodies to the Golgi marker p115 [(a)(i)-(iii)]. The proportions of cells correctly extending lamellipodia into the wound space, with nucleus correctly localised to the rear of the cell, and with Golgi apparatus correctly localised to the 120° sector in front of the nucleus, facing the wound were calculated [(a)(iv)]. The wound-healing experiment was repeated, and cells in the front row of wounds were injected with the marker Alexa594-Dextran (red) in conjunction with (b) the pRK5myc empty vector or with (c) RFP-PAK:CRIB, (d) pRK5myc-PTEN or (e) PTEN-C2. (f) Cells were fixed and stained 4 hours after wounding, and the proportion of cells with correctly re-oriented Golgi apparatus (α-p115, green) was calculated. Statistical significance was tested using a *t*-test (two-sample, heteroskadistic) in Microsoft Excel.

Chapter 4:

Discussion

4.1. Inhibition of Migration by the PTEN C2 Domain

4.1.1. Current Models of PTEN-C2 Activity

The comparatively recent study by *Raftopoulou et al.* showed PTEN to possess an anti-migratory effect in U373 glioma cells, that was unexpectedly attributable to the isolated C2 domain of PTEN (Raftopoulou et al., 2004). Interestingly, it had previously been shown that PTEN was able to exert an anti-migratory effect on cells, and that the lipid phosphatase activity of PTEN was not required for this activity (Tamura et al., 1999;Tamura et al., 1998;Maier et al., 1999). Indeed, PTEN -/- MEFs show an increased rate of migration which is reversible upon re-introduction of PTEN (Tamura et al., 1998). A thesis by *Raftopoulou* reports similar findings in the context of various human glioma cell lines; in characterising the migratory characteristics of U87, U138 and U373 cells, *Raftopoulou* reports that in comparison with astrocytes - from which glioma cells derive - the migration of glioma cells is in general faster and less regulated; these cell lines do not express functional PTEN proteins, and re-introduction of human PTEN into such cells in a wound healing assay shows that migration is inhibited (Raftopoulou, 2003).

The model proposed by *Raftopoulou et al.* (Fig. 1.8) is intriguing and, on the basis of the experimental data provided, convincing; it raises, however, some interesting questions regarding the nature and mechanism of such an anti-migratory effect.

4.1.2. Mechanism of Inhibition of Migration - The Wound-healing Assay and the U373 Glioma System

An initial priority of this project was to further the work of *Raftopoulou* by analyzing the ability of further PTEN constructs to inhibit migration of U373 cells. The wound-healing assay of cell migration employed in those studies occupied a position central to this plan, and so it was attempted to repeat the results of *Raftopoulou* in order to validate this technique for use in the current project. The initial criteria for the assay were identical to those of *Raftopoulou*, with only cells in the row immediately adjacent to the wound at the close of the assay classified as not having been inhibited in migration. The experiments performed clearly show that in the context of this project, the wound-healing assay of cell migration provides results of an inconsistency which would seriously hamper the drawing of solid conclusions regarding the effect of previously untested constructs of cell migration. The experimental work represented by the data in Table 3.1 suggest that, relative to the negative control GFP, only the isolated PTEN C2 domain was able to inhibit migration of U373 cells effectively (Table 3.1). Wild-type PTEN did not inhibit the migration of U373 cells relative to GFP, while the isolated C2 domain inhibited migration to the extent that the proportion of cells found adjacent to the wound was halved (Table 3.1). These results were significantly different from those obtained by *Raftopoulou* ($p = 2.19\times10^{-9}$; $\chi^2=43.24$, 3 degrees of freedom).

It was hypothesised that one reason which could account for the lack of correspondence between these datasets could be that, given the somewhat stochastic dynamics of the monolayer, some cells might be uninhibited with regard to their migration, but reach, for example, only the second or third row of cells at the close of the assay, and thus be perhaps mistakenly classified as having been inhibited in migration. As a result, the experiment was repeated with criteria where the distance between U373 cells microinjected with PTEN or control constructs and the wound-edge 18 hours post-wounding was measured, and mean distances from the wound-edge were calculated, to provide a more

continuous, objective definition of "inhibited migration". It was expected that cells injected with RFPF, the negative control, would give a mean distance corresponding to cells whose migration had not been inhibited, and that constructs having been shown previously to inhibit migration, such as PTEN, PTEN-C2 or N17Rac (Raftopoulou, 2003), would give mean distances significantly higher than the negative control. However, mean distances for the negative control RFPF, the isolated PTEN-C2 domain, the PTEN C-terminus and the catalytically dead PTEN-C124S were comparable, in the order or 140 – 150 μm; conversely, the mean distance from the wound edge of cells microinjected with wild-type PTEN was 70 μm, suggesting that the migration of RFPF-expressing cells was inhibited to a greater extent than those expressing wild-type PTEN (Fig. 3.1). This experiment also suggests that there are inconsistencies between the experiments carried out (Table 3.1, Fig. 3.1). Further, as evidenced by a histogram of distances of individual cells microinjected with PTEN from the wound edge, the range of distances was wide (Fig. 3.1b). Again, the source of this variability is unknown.

The conclusion on the basis of these data was that using the criteria employed in both experiments attempted here (Table 3.1, Fig. 3.1), it was not possible to repeat the results demonstrated by *Raftopoulou*, with regard to the effect of PTEN or control constructs upon migration of U373 cells (Raftopoulou, 2003). As a result, it was not possible to use the wound-healing assay to assess the effect of novel, untested constructs upon the migration of these cells. The reason for the discrepancies both within the results obtained here, and between these results and those reported by *Raftopoulou* is not clear, since experimental conditions were identical.

Clearly, a deeper understanding of the mechanism of action of the PTEN C2 domain in inhibition of migration requires the analysis of the effects of expression of other PTEN constructs on cell migration, and thus a functional migration assay. A number of alternative assays could be possible, and may in

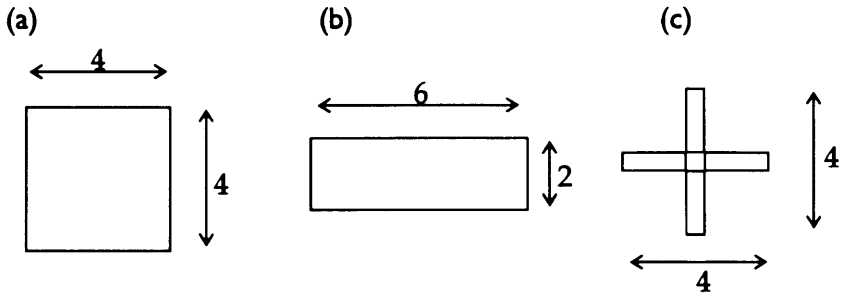
fact possess distinct advantages over the wound-healing assay. Although the wound-healing assay is potentially useful for establishing the existence of an anti-migratory effect in those cells micro-injected with PTEN, relative to non-injected cells, it does not permit further analysis of the nature of the effect. It is thus not clear from the data of *Raftopoulou* (Raftopoulou et al., 2004;Raftopoulou, 2003), for example, whether PTEN-expressing glioma cells are entirely immotile, or whether the rate of migration is merely slowed. Nor is it clear if cell migration is even slowed; whilst this seems the most probable cause, it is possible that cell migration in fact occurs at a normal rate, but that directionality is impaired. In order to establish the nature of migration of PTEN-expressing cells, it would perhaps be insightful to subject cells microinjected with PTEN or PTEN-C2 (and a fluorescent dextran marker) to time-lapse microscopy, such that their migratory behaviour could be more closely examined; it would thus be possible to examine such factors as the polarisation of cell protrusions, and the general direction and rate of migration. This experiment could be performed using sub-confluent cells, or could alternatively involve time-lapse imagine of cells in the context of a monolayer, and could provide insights into the type of signalling pathways implicated in inhibition of migration by C2, based on the cellular behaviours affected. Sub-confluent migration was found to occur in U373 cells (section 3.1) at a rate of 0.329 μm/min (± 0.017, 95% confidence interval) and use of an assay based upon this could provide distinct advantages in analysis of migration, such as visibility of protrusions. However, one potential difficulty could be establishing directionality of movement; this is induced in the context of a wound-healing assay by scratching, but would have to be imposed by some soluble signal in the context of sub-confluent migration. To date, no information is available concerning the response of U373 cells to chemotactic gradients, and the behaviour of these cells in response to a chemical gradient would potentially have to be thoroughly investigated in order to establish an assay based on sub-confluent migration. Another possibility would be to use U373 cell lines stably transfected with relevant PTEN constructs. Providing such cell lines remained

capable of forming a monolayer, a wound-healing experiment could be performed where the monolayer was scratched, and the kinetics of wound-closing could be recorded by time-lapse imaging. This would, again, permit both the determination of constructs' ability to inhibit migration in a continuous, objective way, and also a more detailed analysis of features such as cell protrusion. Clearly, this would require the use of a high-efficiency method of introduction of genes, such as a retroviral system, followed by stringent antibiotic selection for transfected cells.

PTEN expression is lost in a high proportion of tumours, generally leading to a higher rate of cell migration (Yamada and Araki, 2001;Tamura et al., 1998). The U373 cells employed in this work do not express PTEN protein (Raftopoulou, 2003). Whilst this abnormal migration has previously been attributed to loss of PTEN phosphatase activity, it is possible that it is also due in part to the loss of the anti-migratory activity of PTEN-C2. Yet although re-introduction of PTEN into cells deficient for PTEN expression demonstrates that PTEN expression inhibits cellular motility, it is not clear whether this would also be the case if PTEN were to be, for example, over-expressed in cells wild-type for PTEN. Thus it is not apparent whether the migration of cells wild-type for PTEN is occurring at a basal rate, or whether further PTEN expression would decrease migration still further. The investigation of the effect of the C2 on migration of cells wild-type for PTEN, such as mouse embryonic fibroblasts (MEFs) would potentially be profitable. Firstly, conclusions could be drawn regarding the effect of PTEN constructs on cell migration under conditions more closely physiological than those of a cancer cell line; secondly, the use of a cell type such as fibroblasts, whose migratory behaviour is somewhat better characterised than that of, say, U373 glioma cells, would perhaps facilitate a closer analysis of the effect of the PTEN C2 domain on cell migration and protrusion.

4.1.3. Significance of Morphological Changes Caused by PTEN-C2 Expression

Analysis of the morphology of U373 glioma cells nucleofected either with a control vector or with PTEN or PTEN-C2 showed that some distinct morphological differences can be observed (Fig. 3.5); again, these changes did not appear to depend upon the phosphatase activity of PTEN, and were thus obtained even upon expression of the isolated C2 domain. Cell surface area appeared to be diminished (although this difference was not statistically significant - Fig. 3.5a); cell perimeter was remarkably constant (Fig. 3.5b), but the decreased surface area of PTEN/PTEN-C2 expressing cells resulted in a decreased surface area:perimeter ratio, which was statistically significant (Fig. 3.5c). Modelling suggests that this difference could be accounted for in one of two main ways (Figure 4.1).



Perimeter	16 units	16 units	16 units
SA	16 units	12 units	3.75 units
Long. leng.	1	3	1

Figure 4.1:
Modelling shape changes in U373 cells. Various morphological changes can be modelled, allowing changes in computed morphological parameters (such as cell perimeter, surface area, surface area:perimeter ratio, ratio of longest lengths) to be compared with the changes observed in U373 cells, allowing speculation about the cell morphological consequences of these changes. In this rather crude model, (a) represents the equivalent of an unmanipulated cell, whereas (b) and (c) represent morphologies resulting from different methods of manipulating the morphological parameter. SA: surface area; Long. leng.: ratio of longest lengths.

Firstly, cells expressing PTEN-C2 could assume a generally more elongated morphology than control cells (Fig. 4.1b). Secondly, PTEN-C2-expressing cells could undergo more extended protrusion than control cells (Fig. 4.1c). However, Metamorph analysis of U373 glioma cells showed that both in control cells expressing an empty vector, and in cells expressing either PTEN or PTEN-C2, the ratio of longest lengths was not significantly different (Fig. 3.5). This suggests that the hypothesis suggested in Fig. 4.1b is in fact incorrect, since in this case, the ratio of longest lengths would be greatly increased. The hypothesis suggested in Fig. 4.1c is however possible, since the perimeter and the ratio of longest lengths are unchanged compared to the hypothetical control cell, whilst the surface area is significantly decreased. This model could explain the nature of the morphological changes measured in U373 glioma cells expressing PTEN or PTEN-C2. Since the perimeter is unaltered, PTEN-C2-expressing cells would be predicted to have, on average, smaller cell bodies than control cells, as well as more extensive, narrow protrusions. Further, since the ratio of longest lengths is also constant, these protrusions would be predicted to be evenly distributed around the cell body. Such a distribution is perhaps suggestive of a polarity defect. Extensive characterisation of the morphological phenotype of U373 cells is needed to address this question definitively.

The identity of the mechanism responsible for the morphological changes suggested is unknown at this time, yet several possible mechanisms exist. The constancy of cell perimeter both in the case of control cells and in the case of cells expressing PTEN/PTEN-C2, whilst a somewhat crude measure, would appear to indicate that the effect probably does not involve membrane biogenesis. One possibility is that there could be a general up-regulation of protrusion at the level of the cytoskeleton, whilst membrane perimeter would remain constant. How this would be brought about is unclear, but this would have the demonstrated effect of necessarily reducing cell surface area. Rather than merely an increased level of protrusion, it is also perhaps likely that over-

expression of PTEN-C2 could somehow perturb protrusion polarity, resulting in protrusions all around the cell. Given the constant value of the ratio of longest lengths, this seems perhaps to be a most likely hypothesis, but one whose precise mechanistic details remain to be characterised.

4.1.4. Effect of PTEN-C2 on Golgi Polarity in U373 Cells

Preliminary experiments determined that, with respect to the translocation of the nucleus to the rear of the cell (Gomes et al., 2005) and reorientation of the Golgi apparatus (Nobes and Hall, 1999), U373 cells are able to polarize correctly, in response to wounding of a monolayer (Fig. 3.6a). This is a novel finding, and suggests that the level of transformation of the U373 cell line is low. In addition, the proportion of non-injected cells polarizing correctly at 4h (Fig. 3.6a(iv)) is comparable to the proportion of cells injected with Alexa594-Dextran and pRK5myc control vector at the same time point (Fig. 3.6f). This suggests that neither the microinjection technique nor the introduction of the fluorescent dextran or DNA *per se* demonstrates an adverse effect upon the correct polarization of the Golgi apparatus. However, expression of PTEN or PTEN-C2 in U373 cells perturbs their polarity (Figure 3.6f). Ordinarily, in the context of a wound-healing assay, some 75% of leading edge U373 cells orient their Golgi apparatus in front of the nucleus, in the 120° sector facing the wound (Cau and Hall, 2005)(Fig. 3.6f). In cells injected with the PAK-CRIB fragment, known to perturb Golgi polarity, only approximately 50% of cells polarised correctly (Fig. 3.6f). Whilst this perhaps does not appear to be a significant reduction, it is necessary to recall that even if Golgi reorientation were completely random, the expected proportion of cells with correctly orientated Golgi apparatus would be 33%. Interestingly, both PTEN and PTEN-C2 are also able, in the context of a wound-healing assay, to perturb the correct reorientation of the Golgi apparatus almost as well as RFP-PAK-CRIB (Fig. 3.6d-f).

The involvement of PTEN itself in the determination of polarity is not a novel finding, and has been previously demonstrated in the context of chemotaxis in neutrophils and *Dictyostelium* (Iijima and Devreotes, 2002). There, PTEN plays a key role in polarity determination since it is localised uniquely to the back of the cell, and hence PIP_3 is efficiently removed from that region, facilitating a polarised distribution of the lipid to the front of the cell (Iijima and Devreotes, 2002). If PTEN were overexpressed in U373 cells, then it might be expected that this would perturb cell polarity, since the mislocalisation of PTEN throughout the cytosol would lead to the disruption of the PIP_3 gradient. Again, what is particularly surprising is that PTEN is able to bring about this effect upon cellular polarity in U373 cells through the activity of its isolated C2 domain (Fig. 3.6e,f).

It is not clear whether the disturbance of correct reorientation of the Golgi, and hence potentially some disturbance of correct microtubule dynamics, is what is in effect responsible for the anti-migratory effect of the C2 domain in U373 cells; time lapse microscopy of a migrating cells marked with a fluorescent dextran marker and stained with the live cell Golgi-marker Bodipy in the context of a wound-healing assay would be required in order to investigate this hypothesis.

The precise mechanism by which expression of the PTEN C2 domain can exert an effect on cellular polarity is not known. One possibility is through interaction with another protein, implicated in the control of cellular polarity, in a way similar to the migration-inhibitory model proposed in Fig 1.8. Interestingly, a comparatively recent study has identified the kinase LKB1 as an interactor of the PTEN C2 domain (Mehenni et al., 2005). Although LKB1 has been implicated in epithelial polarity mechanisms rather than the polarity issue raised here, it is possible that LKB1 could play some role in the establishment of Golgi polarity. Interestingly, LKB1 has also been shown to phosphorylate PTEN, but the phosphorylation site has not been properly characterised (Mehenni et al., 2005). In order to determine whether this interaction could be of

significance to the anti-migratory phenomenon exhibited by the PTEN C2 domain, it would first be necessary to establish that LKB1 expression has not been lost in U373 cells. Next, a series of immunoprecipitation experiments with various PTEN mutants, following the range employed by *Raftopoulou et al.* when initially characterising the effect, would allow discernment of whether LKB1/C2 interaction data correlated with anti-migratory effects. An interesting possibility is that LKB could phosphorylate Thr 383; this could be tested by mass spectrometry, by Western blotting using phospho-Thr383-specific antibodies, or by combining ^{32}P labelling with mutagenesis of Thr383 in an *in vitro* phosphorylation assay.

Much work has shown that the role of PTEN in maintaining a PIP_3 or (by inference) $PI(4,5)P_2$ gradient is crucial for proper directionality of movement in neutrophils and *Dictyostelium* cells (Devreotes and Janetopoulos, 2003;Iijima et al., 2002;Iijima and Devreotes, 2002;Janetopoulos et al., 2004). If the PIP_3 gradient in cells is implicated in proper re-orientation of the Golgi apparatus (and therefore also of the MTOC), another possibility is that the PTEN C2 domain disrupts proper cell polarity by sequestering lipids, and so by effectively abolishing the gradient. If this were found to be the case, then a necessary consequence would be that the polarity-disrupting effect of expression of the C2 domain could in no sense be responsible for C2's anti-migratory effect, since the synaptotagmin C2B domain, which binds - and thus potentially sequesters - the same inositol phospholipids as PTEN-C2, is unable to abrogate migration of U373 cells in a wound-healing assay (Raftopoulou et al., 2004); this mechanism thus seems unlikely, but remains possible, and could be tested by repeating the wound healing/polarity assay with the synaptotagmin C2 domain.

4.2. Bioinformatics of PTEN-family Proteins

Sequence alignment techniques demonstrate that the PTEN C2 domain shows relatively little primary sequence homology with domains from other members

of the PTEN gene family (Fig. 3.2a). Assuming a common ancestral gene, this suggests that the gene duplication event which led to the divergence of PTEN from other family members was relatively early. Members of the TPTE/TPIP families are more closely related to each other, suggesting that a similar gene duplication event resulting in the divergence of TPTE from TPIP is comparatively more recent. Comparison of the 3D structures (Fig. 3.2b,c) showed that for the C2 domains at least, primary sequence homology is not a good predictor of structural or functional homology. It is therefore not clear whether TPTE/TPIP family members possess a C2 domain that might also exert an anti-migratory effect in U373 cells. Since some TPIP family members have been found to be expressed in brain tissue, it would perhaps be of interest to determine whether these are expressed in U373 glioma cells, and to determine their anti-migratory activity in the context of the wound-healing assay. Since no TPIP/TPTE family member except TPIPα has been shown to possess any phosphatidylinositol phosphatase activity, the presence of a functional C2 domain could suggest a novel function for these proteins (Tapparel et al., 2003;Guipponi et al., 2001).

Despite little overall sequence homology, several residues do emerge from sequence alignment as being conserved across the PTEN, TPTE and TPIP families. This raises the possibility that these residues may be of some importance for PTEN-C2-domain function. A potentially fruitful approach could be to observe the effects of mutagenesis of these residues on the anti-migratory effect of the C2 domain.

4.3. Significance of Phosphoinositide-Binding by PTEN-C2

One function of C2 domains from other proteins, such as the prototypical Ca-dependent C2 domains of PKC or the Ca-independent C2 domains of PI3K or PLCδ, is membrane binding (Murray and Honig, 2002;Nalefski and Newton, 2001). Since the PTEN C2 domain has been implicated in membrane binding (Georgescu et al., 2000), it was hypothesised that it might also be capable of

binding, and thus potentially sequestering, signalling lipids, such as phosphatidylinositols, and thereby lead to an inhibition of cellular migration. N-terminal fusions of GST with the C2 domain of PTEN or the C2B domain of synaptotagmin we successfully sub-cloned and expressed in and purified from *E. coli* cells (Fig. 3.3a-d). Comparison of the lipid-binding profile of the migration-inhibiting PTEN C2 domain with the non-inhibitory synaptotagmin C2B domain showed that both domains bound an identical spectrum of lipids (Figure 3.3e-g), suggesting that lipid-binding is unlikely to be the cause of anti-migratory activity. In addition, the accuracy of the PIPStrip assay carried out was supported by data showing that isolated GST did not bind to any lipid on the strip (Fig. 3.3e) and that the lipid binding specificities of both PLCδ-PH domain and the Hrs1-FYVE domain, both of which have been well defined (Yu and Lemmon, 2001), were confirmed by the PIPStrip assay (Fig. 3.3h,i).

However, it is interesting to note that this experiment revealed a hitherto uncharacterised lipid-binding activity of PTEN C2. Whilst the concept of the C2 domain being implicated in membrane- and hence lipid-binding is not new, what is of interest is the lipid specificity of PTEN-C2. Whilst it appears to bind phosphatidylinositols in a somewhat promiscuous fashion, binding PI-3-P, PI-4-P, PI-5-P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, PI(3,4,5)P_3, the PTEN C2 domain does not bind unsubstituted PI, nor indeed any non-inositol-based membrane lipid.

The significance of lipid- or membrane-binding for the PTEN C2 domain is unclear, both in the context of the naked C2 domain and its anti-migratory effect, as reported by *Raftopoulou et al.*, and in the context of the full-length physiological protein. It was initially believed that the C2 domain of PTEN was crucially important for either membrane-binding, or for molecular "fine-tuning" at the membrane, where it was suggested that membrane-C2 interactions would be of crucial importance for the correct orientation of the active site of PTEN relative to its lipid substrates in the membrane (Georgescu

et al., 2000). Subsequently a study employing SPR to measure affinities of PTEN domains for membrane-mimicking vesicles concluded that the affinity of PTEN-C2 for such vesicles is not great enough to provide the energetic driving force for membrane recruitment (Das et al., 2003). However, one problem with the methodology employed by this study is that the membrane-mimicking vesicles consisted only of a mixture of substituted phosphatidylserine and substituted phosphatidylcholine; however the result that the affinity of PTEN-C2 for such vesicles is remarkably weak is perhaps not surprising, since on the basis of the results presented here (Fig. 3.3), the C2 domain of PTEN does not interact in any significant way with either PC or PS. These data somewhat weaken the conclusion of *Das et al.* regarding the energetic role of PTEN-C2 in membrane-binding; it is possible that PTEN-C2 is more important for membrane-recruitment of the PTEN molecule than suggested by their study, but that such high-affinity binding would take place only at local sites of inositol phospholipid concentration. This is perhaps intuitively likely, since it would at once represent a method of selectively recruiting PTEN to the cell membrane, where such lipids are prevalent, and a method of recruiting the PTEN molecule to sites where its substrates (D3-phosphorylated phosphatidylinositols) are in abundance. As a result, the C2 domain of PTEN may, together with the N-terminal PIP_2-binding motif, function *in vivo* to regulate the selectivity of PTEN membrane-recruitment.

Assuming that PTEN-C2 does indeed engage in membrane-binding *in vivo*, this would perhaps suggest that the sub-cellular location of any anti-migratory signalling would be at the cell membrane. It is possible that upon recruitment to the plasma membrane, the PTEN C2 domain could interact with a protein implicated in positive regulation of cell motility in such a way as to abrogate its signal. Indeed, the PTEN molecule both binds membrane lipids (Das et al., 2003) and has previously been shown to bind a number of membrane proteins (Wu et al., 2000a;Wu et al., 2000b), making a membrane site seem perhaps particularly likely.

Although no data are known for the PTEN-C2 domain, examples from other C2 domains suggest that the C2 domain may be able to regulate cell signalling in intriguing ways hitherto uncharacterised. A relatively recent study found that the C2 domain of PLCδ was able to interact with phosphotyrosine residues of receptors, thus modulating receptor tyrosine kinase signalling (Benes et al., 2005); similarly this C2 domain had already been described as an actin-binding domain (Lopez-Lluch et al., 2001). If the C2 domain of PTEN were also able to bind similar targets, this might represent one way in which it might effect its anti-migratory signalling. Equally, it would perhaps be interesting to observe, by means of a wound-healing assay, the effects on cell motility in U373 cells of microinjecting (for example) PLCδ-C2.

4.4. Regulation of the Anti-migratory Activity of PTEN-C2

4.4.1. Regulation of Sub-cellular Localisation of PTEN

In the context of the full-length PTEN protein, phosphorylation of Thr383 is able to regulate the ability of the C2 domain to inhibit cell migration. One potential way in which this might occur is by regulation of the sub-cellular distribution of PTEN; whilst phosphorylation-prompted redistribution could either take place at the level of a protein complex, where phosphorylation would abrogate (or indeed promote) a protein-protein interaction, this could also occur at the sub-cellular level, with phosphorylation regulating the transport of PTEN into or out of the nucleus, or some other sub-cellular compartment. Experiments in transfected Cos7 cells in fact showed that the T383D mutant PTEN was predominantly localised to the cytosol, whereas the T383A mutant was predominantly nuclear (Fig. 3.4). The T383A/D mutant constructs used were also catalytically dead (C124S) mutants, and so the possibility that the differential sub-cellular localisation could be due to PTEN phosphatase activity is eliminated.

Although it is not possible to draw conclusions from the data generated by these experiments concerning the mechanism of nuclear translocation, several potential mechanisms exist. Possibly the most likely method would be via association with Major Vault Protein. A recent study (Chung and Eng, 2005) has shown that PTEN contains an unconventional NLS-like sequence, which is capable of regulating nuclear/cytoplasmic partitioning of the PTEN protein via association with major vault protein. Such "piggyback" mechanisms are emerging as a common mechanism of nuclear/cytosolic transport of proteins which themselves lack appropriate sequences. A common example would be the mdm2 protein, whose nuclear/cytoplasmic localisation is determined almost exclusively by interaction with β–arrestins, which possess nuclear localisation/export sequences (Wang et al., 2003). This hypothesis could be tested by conducting immunoprecipitation experiments between major vault protein and PTEN constructs with the T383A or T383D mutations.

Whilst it is also tempting, based on the data presented here, to suggest that regulation of nuclear/cytoplasmic localisation of PTEN is entirely dependent on Thr383 phosphorylation, previous data presented in the context of wound-healing assays has suggested that mutation of any of a number of phosphorylatable residues in the C-terminal tail (namely Ser380, Thr382, Thr383 or Thr385) to alanine is sufficient to cause expression of myc-tagged PTEN constructs to become predominantly nuclear (Raftopoulou, 2003). One possible explanation for this is that sub-cellular localisation of PTEN is regulated by overall charge in this C-terminal region, rather than by the phosphorylation of particular residues.

Localisation of PTEN to the nucleus is in itself not a new finding (Lian and Di, 2005), although regulation of this localisation by phosphorylation of the C-terminal tail has not been previously described. Differential localisation of PTEN at various points of the cell cycle has however been reported (Ginn-Pease and Eng, 2003), but it is not clear whether, for example, the varying sub-cellular

localisation of PTEN is itself due to a cell cycle-induced phosphorylation/dephosphorylation at Thr383 or indeed whether, conversely, phosphorylation at Thr383 is regulated by sub-cellular localisation. It would perhaps be valuable to determine the phosphorylation status of C-terminal residues throughout the cell cycle in synchronised cells.

Given the somewhat clear distinction between the ability of T383A/D mutants to inhibit cell migration in U373 cells, it is tempting to speculate that since T383A mutants are predominantly localised to the nucleus, the signalling event resulting in inhibition of migration must occur in the nucleus. A complicating feature of this model is that the naked C2 domain, which is also able to inhibit cell motility, is predominantly localised not to the nucleus, but to the cytosol. This is not, however, to suggest that these data are irreconcilable; it is possible that due to its relatively small size (~ 20 kDa), PTEN-C2 is able to passively diffuse into and out of the nucleus, and so, while it is does not accumulate there, it is still able to signal in the same way as the predominantly nuclear PTEN-CS-T383A and hence inhibit migration. Perhaps the best way of testing a sub-cellular localisation-dependent hypothesis of migration inhibition would be to test the ability of the PTEN-C2 domain fused either to a known NLS or to a CAAX-box to inhibit cell migration in the context of the wound-healing assay.

Were a nuclear signalling event responsible for the inhibition of migration caused by PTEN-C2, one possibility is that C2 could in some way either up- or down-regulate the transcription of genes whose protein products are implicated in control of cell motility. Certainly, a recent study where 2D gel electrophoresis analysis was used to quantify changes in expression levels of proteins when PTEN was expressed in U87 cells demonstrated that both phosphatase-dependent and -independent modulation of gene transcription occurred as a result of PTEN expression (Kim et al., 2003).

Interestingly, *Raftopoulou* found that in U87 cells, expression of the C2 domain alone was not sufficient to inhibit cell migration, and the PDZ-binding domain was needed in addition. The PDZ-binding motif has been implicated in the binding of PTEN to a number of membrane proteins (Kotelevets et al., 2005;Tolkacheva et al., 2001), perhaps suggesting that the site of C2-domain signalling may in fact be the plasma membrane. Clearly, the question of the sub-cellular environment in which C2 signals to inhibit migration remains open.

4.4.2. Regulation of the PTEN C2 Domain

Raftopoulou et al. propose an intriguing model of regulation of the anti-migratory activity of PTEN by phosphorylation of the PTEN C-terminal tail at Thr383 (Figure 1.8). The suggestion is that such regulation may proceed via modulation of PTEN conformation, such that when phosphorylated, the PTEN tail would engage in an intramolecular interaction, such that the C2 domain would be shielded from interacting with its binding partner. Further, interconversion between active and inactive conformations (with respect to the C2 domain) would be possible according to this model by cycles of dephosphorylation and phosphorylation of Thr383. Again, as intriguing a model as this is, a number of questions concerning its precise details remain.

Firstly, the identities of the kinase and phosphatase responsible for modulation of the phosphorylation state are not currently known. Although it has been proposed that the CK2 kinase is responsible for phosphorylation of Thr383 this has been put into some question by another study (Li et al., 2005;Torres and Pulido, 2001). Furthermore, although catalytically dead PTEN is not able to inhibit motility when injected into U373 cells in a wound-healing assay, mutation of Thr383 to alanine is capable of rescuing this ability even in catalytically dead PTEN (Raftopoulou, 2003;Raftopoulou et al., 2004). This intriguing piece of data led *Raftopoulou et al.* to speculate that this pointed to an autodephosphorylation at Thr383 by PTEN itself. Given this result this seems

both possible and likely; indeed, the relatively acidic context in which Thr383 is situated conforms to the PTEN consensus described elsewhere (Tamura et al., 1998). The result that exogenous PTEN must retain catalytic activity towards protein substrates in order for the C2 domain to successfully inhibit cell migration in the context of the full protein strongly suggests that PTEN could exercise an autocatalytic activity towards phosphorylated Thr383. It must however be stressed that this is not the only interpretation of these results; an alternative hypothesis could be that PTEN could dephosphorylate, and activate, a separate protein phosphatase, which would then dephosphorylate Thr383 (Figure 4.2).

Secondly, Thr383 lies among a complex array of other phosphorylatable residues in the C-terminal tail of PTEN. Experiments carried out by *Raftopoulou et al.* appear to show that Thr383 is however the major residue for regulation of the anti-migratory signalling of the C2 domain (Raftopoulou et al., 2004). Single mutation of this residue either to a non-phosphorylatable alanine residue or to a phosphomimetic aspartate residue demonstrates that at this site alone is modulation of phosphorylation state able to affect the ability of the C2 domain to inhibit cell motility. However, some questions remain. For example, it is not clear whether phosphorylation of Thr383 is an independent event, or whether this is perhaps mechanistically linked to the phosphorylation of other residues in the 380-385 region of C-terminal tail. Certainly, regulation of phosphorylation of upstream residues has been shown to be dependent on

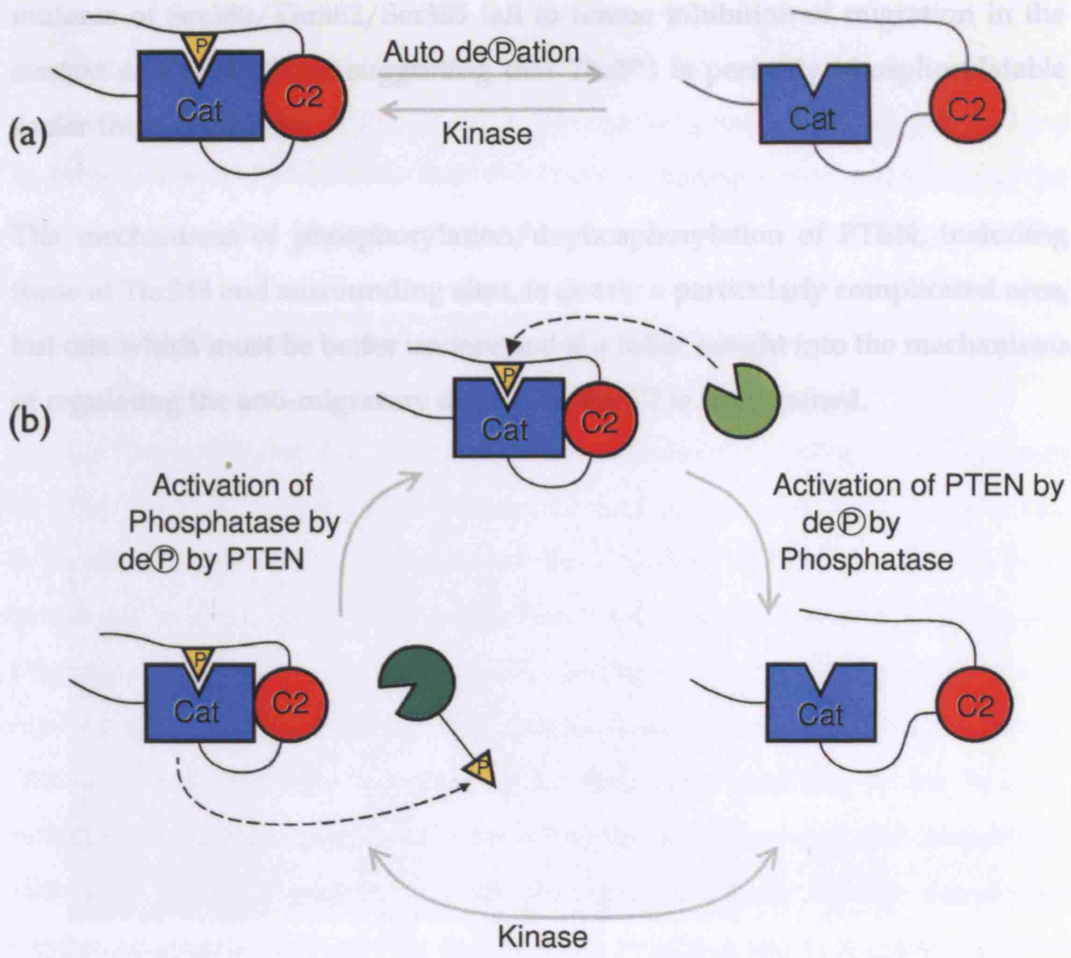


Figure 4.2:
Models of dephosphorylation of Thr383. PTEN could perform an autodephosphorylation at this site (a), or could alternatively dephosphorylate (and activate) a phosphatase (green), which would proceed to dephosphorylate PTEN, and so activate C2-domain anti-migratory signalling (b).

priming by phosphorylation (Al-Khouri et al., 2005) and it is possible that this could also apply at Thr383. For example, as detailed above, data from *Raftopoulou* suggest that mutation of any residue out of Ser380/Thr382/Ser385 results in a PTEN species whose sub-cellular localisation is predominantly nuclear; clearly, a single mutation at one phosphorylatable site does not control for the phosphorylation state of the other sites, and so it is possible that the nuclear localisation of Ser380/Thr382/Ser385 mutants occurs since these mutations somehow impair the phosphorylation of Thr383. However, it would

be difficult to integrate such a model with the finding that the same single mutants of Ser380/Thr382/Ser385 fail to rescue inhibition of migration in the context of PTEN-C124S, suggesting that Thr383 is perfectly phosphorylatable under these conditions.

The mechanisms of phosphorylation/dephosphorylation of PTEN, including those at Thr383 and surrounding sites, is clearly a particularly complicated area, but one which must be better understood if a fuller insight into the mechanisms of regulating the anti-migratory activity of the C2 is to be gained.

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Christo omnino nihil praeponant, qui nos pariter ad vitam aeternam perducat.

אָכֵן יֵשׁ יְהוָה בַּמָּקוֹם הַזֶּה ; וְאָנֹכִי, לֹא יָדָעְתִּי.

ἵνα ἐν πᾶσιν δοξάζηται ὁ θεὸς διὰ Ἰησοῦ Χριστοῦ, ᾧ ἐστιν ἡ δόξα καὶ τὸ κράτος εἰς τοὺς αἰῶνας τῶν αἰώνων: ἀμήν.

References

1. Accili, D. and K.C.Arden. 2004. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell.* 117:421-426.

2. Adey, N.B., L.Huang, P.A.Ormonde, M.L.Baumgard, R.Pero, D.V.Byreddy, S.V.Tavtigian, and P.L.Bartel. 2000. Threonine phosphorylation of the MMAC1/PTEN PDZ binding domain both inhibits and stimulates PDZ binding. *Cancer Res.* 60:35-37.

3. Al-Khouri, A.M., Y.Ma, S.H.Togo, S.Williams, and T.Mustelin. 2005. Cooperative phosphorylation of the tumor suppressor phosphatase PTEN by casein kinases and glycogen synthase kinase 3beta. *J. Biol. Chem.* 280:35195-35202.

4. Arnaout, M.A., B.Mahalingam, and J.P.Xiong. 2005. Integrin structure, allostery, and bidirectional signaling. *Annu. Rev. Cell Dev. Biol.* 21:381-410.:381-410.

5. Baeza, N., M.Weller, Y.Yonekawa, P.Kleihues, and H.Ohgaki. 2003. PTEN methylation and expression in glioblastomas. *Acta Neuropathol. (Berl)* 106:479-485.

6. Benes, C.H., N.Wu, A.E.Elia, T.Dharia, L.C.Cantley, and S.P.Soltoff. 2005. The C2 domain of PKCdelta is a phosphotyrosine binding domain. *Cell.* 121:271-280.

7. Bernards, A. 2003. GAPs galore! A survey of putative Ras superfamily GTPase activating proteins in man and Drosophila. *Biochim. Biophys. Acta.* 1603:47-82.

8. Bertoglio, J.H., L.Rimsky, E.S.Kleinerman, and L.B.Lachman. 1987. B-cell line-derived interleukin 1 is cytotoxic for melanoma cells and promotes the proliferation of an astrocytoma cell line. *Lymphokine Res.* 6:83-91.

9. Borowsky, M.L. and R.O.Hynes. 1998. Layilin, a novel talin-binding transmembrane protein homologous with C-type lectins, is localized in membrane ruffles. *J. Cell Biol.* 143:429-442.

10. Burgering, B.M. and G.J.Kops. 2002. Cell cycle and death control: long live Forkheads. *Trends Biochem. Sci.* 27:352-360.

11. Campbell, R.B., F.Liu, and A.H.Ross. 2003. Allosteric activation of PTEN phosphatase by phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* 278:33617-33620.

12. Cancer Research UK. Mortality and Morbidity of Cancers in the UK. 2005.
Ref Type: Internet Communication

13. Carragher, N.O. and M.C.Frame. 2004. Focal adhesion and actin dynamics:a place where kinases and proteases meet to promote invasion. *Trends Cell Biol.* 14:241-249.

14. Cau, J. and A.Hall. 2005. Cdc42 controls the polarity of the actin and microtubule cytoskeletons through two distinct signal transduction pathways. *J. Cell Sci.* 118:2579-2587.

15. Chang, C.J., D.J.Freeman, and H.Wu. 2004. PTEN regulates Mdm2 expression through the P1 promoter. *J. Biol. Chem.* 279:29841-29848.

16. Chen, H., C.Rossier, M.A.Morris, H.S.Scott, A.Gos, A.Bairoch, and S.E.Antonarakis. 1999. A testis-specific gene, TPTE, encodes a putative transmembrane tyrosine phosphatase and maps to the pericentromeric region of human chromosomes 21 and 13, and to chromosomes 15, 22, and Y. *Hum. Genet.* 105:399-409.

17. Chung, J.H. and C.Eng. 2005. Nuclear-cytoplasmic partitioning of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) differentially regulates the cell cycle and apoptosis. *Cancer Res.* 65:8096-8100.

18. Critchley, D.R. 2005. Genetic, biochemical and structural approaches to talin function. *Biochem. Soc. Trans.* 33:1308-1312.

19. Cully, M., H.You, A.J.Levine, and T.W.Mak. 2006. Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat. Rev. Cancer.* 6:184-192.

20. Das, S., J.E.Dixon, and W.Cho. 2003. Membrane-binding and activation mechanism of PTEN. *Proc. Natl. Acad. Sci. U. S. A* 100:7491-7496.

21. Datta, S.R., H.Dudek, X.Tao, S.Masters, H.Fu, Y.Gotoh, and M.E.Greenberg. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell.* 91:231-241.

22. Davies, M.A., D.Koul, H.Dhesi, R.Berman, T.J.McDonnell, D.McConkey, W.K.Yung, and P.A.Steck. 1999. Regulation of Akt/PKB activity, cellular growth, and apoptosis in prostate carcinoma cells by MMAC/PTEN. *Cancer Res.* 59:2551-2556.

23. del Peso, L., M.Gonzalez-Garcia, C.Page, R.Herrera, and G.Nunez. 1997. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science.* 278:687-689.

24. Devreotes, P. and C.Janetopoulos. 2003. Eukaryotic chemotaxis: distinctions between directional sensing and polarization. *J. Biol. Chem.* 278:20445-20448.

25. Dijkers, P.F., R.H.Medema, C.Pals, L.Banerji, N.S.Thomas, E.W.Lam, B.M.Burgering, J.A.Raaijmakers, J.W.Lammers, L.Koenderman, and P.J.Coffer. 2000. Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). *Mol. Cell Biol.* 20:9138-9148.

26. Domchek, S.M., K.R.Auger, S.Chatterjee, T.R.Burke, Jr., and S.E.Shoelson. 1992. Inhibition of SH2 domain/phosphoprotein association by a nonhydrolyzable phosphonopeptide. *Biochemistry.* 31:9865-9870.

27. Downes, C.P., S.Walker, G.McConnachie, Y.Lindsay, I.H.Batty, and N.R.Leslie. 2004. Acute regulation of the tumour suppressor phosphatase, PTEN, by anionic lipids and reactive oxygen species. *Biochem. Soc. Trans.* 32:338-342.

28. Ehrlich, H.P. 1988. Wound closure: evidence of cooperation between fibroblasts and collagen matrix. *Eye.* 2:149-157.

29. Etienne-Manneville, S. and A.Hall. 1999. Integrin-mediated activation of cdc42 controls cell polarity in migrating astrocytes through PKC-zeta. *Cell* 106:489-498.

30. Faix, J. and K.Rottner. 2006. The making of filopodia. *Curr. Opin. Cell Biol.* 18:18-25.

31. Flynn, P., M.Wongdagger, M.Zavar, N.M.Dean, and D.Stokoe. 2000. Inhibition of PDK-1 activity causes a reduction in cell proliferation and survival. *Curr. Biol.* 10:1439-1442.

32. Freeman, D.J., A.G.Li, G.Wei, H.H.Li, N.Kertesz, R.Lesche, A.D.Whale, H.Martinez-Diaz, N.Rozengurt, R.D.Cardiff, X.Liu, and H.Wu. 2003. PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. *Cancer Cell* 3:117-130.

33. Friedrich, P. and Z.Bozoky. 2005. Digestive versus regulatory proteases: on calpain action in vivo. *Biol. Chem.* 386:609-612.

34. Garcia-Alvarez, B., J.M.de Pereda, D.A.Calderwood, T.S.Ulmer, D.Critchley, I.D.Campbell, M.H.Ginsberg, and R.C.Liddington. 2003. Structural determinants of integrin recognition by talin. *Mol. Cell.* 11:49-58.

35. Georgescu, M.M., K.H.Kirsch, T.Akagi, T.Shishido, and H.Hanafusa. 1999. The tumor-suppressor activity of PTEN is regulated by its carboxyl-terminal region. *Proc. Natl. Acad. Sci. U. S. A* 96:10182-10187.

36. Georgescu, M.M., K.H.Kirsch, P.Kaloudis, H.Yang, N.P.Pavletich, and H.Hanafusa. 2000. Stabilization and productive positioning roles of the C2 domain of PTEN tumor suppressor. *Cancer Res.* 60:7033-7038.

37. Ghosh, A.K., I.Grigorieva, R.Steele, R.G.Hoover, and R.B.Ray. 1999. PTEN transcriptionally modulates c-myc gene expression in human breast carcinoma cells and is involved in cell growth regulation. *Gene* 235:85-91.

38. Giancotti, F.G. and E.Ruoslahti. 1999. Integrin signaling. *Science.* 285:1028-1032.

39. Ginn-Pease, M.E. and C.Eng. 2003. Increased nuclear phosphatase and tensin homologue deleted on chromosome 10 is associated with G0-G1 in MCF-7 cells. *Cancer Res.* 63:282-286.

40. Goberdhan, D.C., N.Paricio, E.C.Goodman, M.Mlodzik, and C.Wilson. 1999. Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. *Genes Dev.* 13:3244-3258.

41. Gomes, E.R., S.Jani, and G.G.Gundersen. 2005. Nuclear movement regulated by Cdc42, MRCK, myosin, and actin flow establishes MTOC polarization in migrating cells. *Cell.* 121:451-463.

42. Guipponi, M., C.Tapparel, O.Jousson, N.Scamuffa, C.Mas, C.Rossier, P.Hutter, P.Meda, R.Lyle, A.Reymond, and S.E.Antonarakis. 2001. The murine orthologue of the Golgi-localized TPTE protein provides clues to the evolutionary history of the human TPTE gene family. *Hum. Genet.* 109:569-575.

43. Gupta, R., J.T.Ting, L.N.Sokolov, S.A.Johnson, and S.Luan. 2002. A tumor suppressor homolog, AtPTEN1, is essential for pollen development in Arabidopsis. *Plant Cell* 14:2495-2507.

44. Hall, A. 2005. Rho GTPases and the control of cell behaviour. *Biochem. Soc. Trans.* 33:891-895.

45. Haus, U., H.Hartmann, P.Trommler, A.A.Noegel, and M.Schleicher. 1991. F-actin capping by cap32/34 requires heterodimeric conformation and can be inhibited with PIP2. *Biochem. Biophys. Res. Commun.* 181:833-839.

46. Hawkins, M., B.Pope, S.K.Maciver, and A.G.Weeds. 1993. Human actin depolymerizing factor mediates a pH-sensitive destruction of actin filaments. *Biochemistry.* 32:9985-9993.

47. Heiss, S.G. and J.A.Cooper. 1991. Regulation of CapZ, an actin capping protein of chicken muscle, by anionic phospholipids. *Biochemistry.* 30:8753-8758.

48. Heneberg, P. and P.Draber. 2005. Regulation of cys-based protein tyrosine phosphatases via reactive oxygen and nitrogen species in mast cells and basophils. *Curr. Med. Chem.* 12:1859-1871.

49. Hettinger, K., F.Vikhanskaya, M.K.Poh, M.K.Lee, B.De, I, J.T.Zhang, S.A.Reddy, and K.Sabapathy. 2006. c-Jun promotes cellular survival by suppression of PTEN. *Cell Death. Differ.* 2006:1-12.

50. Higgs, H.N. and T.D.Pollard. 2001. Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Annu. Rev. Biochem.* 70:649-76.:649-676.

51. Hool, L.C. 2006. Reactive oxygen species in cardiac signalling: from mitochondria to plasma membrane ion channels. *Clin. Exp. Pharmacol. Physiol.* 33:146-151.

52. Hotchin, N.A. and A.Hall. 1995. The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases. *J. Cell Biol.* 131:1857-1865.

53. Hsu, S.C., O.V.Volpert, P.A.Steck, T.Mikkelsen, P.J.Polverini, S.Rao, P.Chou, and N.P.Bouck. 1996. Inhibition of angiogenesis in human glioblastomas by chromosome 10 induction of thrombospondin-1. *Cancer Res.* 56:5684-5691.

54. Humbert, P., S.Russell, and H.Richardson. 2003. Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. *Bioessays.* 25:542-553.

55. Iijima, M. and P.Devreotes. 2002. Tumor suppressor PTEN mediates sensing of chemoattractant gradients. *Cell* 109:599-610.

56. Iijima, M., Y.E.Huang, and P.Devreotes. 2002. Temporal and spatial regulation of chemotaxis. *Dev. Cell* 3:469-478.

57. Isenberg, G. and W.H.Goldmann. 1998. Peptide-specific antibodies localize the major lipid binding sites of talin dimers to oppositely arranged N-terminal 47 kDa subdomains. *FEBS Lett.* 426:165-170.

58. Janetopoulos, C., L.Ma, P.N.Devreotes, and P.A.Iglesias. 2004. Chemoattractant-induced phosphatidylinositol 3,4,5-trisphosphate

accumulation is spatially amplified and adapts, independent of the actin cytoskeleton. *Proc. Natl. Acad. Sci. U. S. A* 101:8951-8956.

59. Katso, R., K.Okkenhaug, K.Ahmadi, S.White, J.Timms, and M.D.Waterfield. 2001. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu. Rev. Cell Dev. Biol.* 17:615-75.:615-675.

60. Keller, R. 2005. Cell migration during gastrulation. *Curr. Opin. Cell Biol.* 17:533-541.

61. Kim, S.Y., Y.S.Kim, and Y.Y.Bahk. 2003. Proteome changes induced by expression of tumor suppressor PTEN. *Mol. Cells* 15:396-405.

62. Knobbe, C.B., A.Merlo, and G.Reifenberger. 2002. Pten signaling in gliomas. *Neuro-oncol.* 4:196-211.

63. Kops, G.J. and B.M.Burgering. 1999. Forkhead transcription factors: new insights into protein kinase B (c-akt) signaling. *J. Mol. Med.* 77:656-665.

64. Kotelevets, L., H.J.van, E.Bruyneel, M.Mareel, R.F.van, and E.Chastre. 2005. Implication of the MAGI-1b/PTEN signalosome in stabilization of adherens junctions and suppression of invasiveness. *FASEB J.* 19:115-117.

65. Koul, D., R.Parthasarathy, R.Shen, M.A.Davies, S.A.Jasser, S.K.Chintala, J.S.Rao, Y.Sun, E.N.Benvenisite, T.J.Liu, and W.K.Yung. 2001. Suppression of matrix metalloproteinase-2 gene expression and invasion in human glioma cells by MMAC/PTEN. *Oncogene* 20:6669-6678.

66. Le, Y., Y.Zhou, P.Iribarren, and J.Wang. 2004. Chemokines and chemokine receptors: their manifold roles in homeostasis and disease. *Cell Mol. Immunol.* 1:95-104.

67. Lee, H.S., R.M.Bellin, D.L.Walker, B.Patel, P.Powers, H.Liu, B.Garcia-Alvarez, J.M.de Pereda, R.C.Liddington, N.Volkmann, D.Hanein, D.R.Critchley, and R.M.Robson. 2004. Characterization of an actin-binding site within the talin FERM domain. *J. Mol. Biol.* 343:771-784.

68. Lee, J.O., H.Yang, M.M.Georgescu, C.A.Di, T.Maehama, Y.Shi, J.E.Dixon, P.Pandolfi, and N.P.Pavletich. 1999. Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell* 99:323-334.

69. Leslie, N.R., D.Bennett, Y.E.Lindsay, H.Stewart, A.Gray, and C.P.Downes. 2003. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *EMBO J.* 22:5501-5510.

70. Li, D.M. and H.Sun. 1997. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res.* 57:2124-2129.

71. Li, D.M. and H.Sun. 1998. PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. *Proc. Natl. Acad. Sci. U. S. A* 95:15406-15411.

72. Li, J., C.Yen, D.Liaw, K.Podsypanina, S.Bose, S.I.Wang, J.Puc, C.Miliaresis, L.Rodgers, R.McCombie, S.H.Bigner, B.C.Giovanella, M.Ittmann, B.Tycko, H.Hibshoosh, M.H.Wigler, and R.Parsons. 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 275:1943-1947.

73. Li, Z., X.Dong, Z.Wang, W.Liu, N.Deng, Y.Ding, L.Tang, T.Hla, R.Zeng, L.Li, and D.Wu. 2005. Regulation of PTEN by Rho small GTPases. *Nat. Cell Biol.* 7:399-404.

74. Lian, Z. and C.A.Di. 2005. Class reunion: PTEN joins the nuclear crew. *Oncogene.* 24:7394-7400.

75. Liliental, J., S.Y.Moon, R.Lesche, R.Mamillapalli, D.Li, Y.Zheng, H.Sun, and H.Wu. 2000. Genetic deletion of the Pten tumor suppressor gene promotes cell motility by activation of Rac1 and Cdc42 GTPases. *Curr. Biol.* 10:401-404.

76. Litchfield, D.W. 2003. Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem. J.* 369:1-15.

77. Liu, G., C.D.Guibao, and J.Zheng. 2002. Structural insight into the mechanisms of targeting and signaling of focal adhesion kinase. *Mol. Cell Biol.* 22:2751-2760.

78. Lopez-Lluch, G., M.M.Bird, B.Canas, J.Godovac-Zimmerman, A.Ridley, A.W.Segal, and L.V.Dekker. 2001. Protein kinase C-delta C2-like domain is a binding site for actin and enables actin redistribution in neutrophils. *Biochem. J.* 357:39-47.

79. Machesky, L.M., S.J.Atkinson, C.Ampe, J.Vandekerckhove, and T.D.Pollard. 1994. Purification of a cortical complex containing two unconventional actins from Acanthamoeba by affinity chromatography on profilin-agarose. *J. Cell Biol.* 127:107-115.

80. Machesky, L.M. and R.H.Insall. 1998. Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr. Biol.* 8:1347-1356.

81. Machesky, L.M., E.Reeves, F.Wientjes, F.J.Mattheyse, A.Grogan, N.F.Totty, A.L.Burlingame, J.J.Hsuan, and A.W.Segal. 1997. Mammalian actin-related protein 2/3 complex localizes to regions of lamellipodial protrusion and is composed of evolutionarily conserved proteins. *Biochem. J.* 328:105-112.

82. Maciver, S.K., B.J.Pope, S.Whytock, and A.G.Weeds. 1998. The effect of two actin depolymerizing factors (ADF/cofilins) on actin filament turnover: pH sensitivity of F-actin binding by human ADF, but not of Acanthamoeba actophorin. *Eur. J. Biochem.* 256:388-397.

83. Maehama, T. and J.E.Dixon. 1998. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* 273:13375-13378.

84. Maehama, T., G.S.Taylor, and J.E.Dixon. 2001. PTEN and myotubularin: novel phosphoinositide phosphatases. *Annu. Rev. Biochem.* 70:247-79.:247-279.

85. Maier, D., G.Jones, X.Li, A.H.Schonthal, O.Gratzl, E.G.Van Meir, and A.Merlo. 1999. The PTEN lipid phosphatase domain is not required to inhibit invasion of glioma cells. *Cancer Res.* 59:5479-5482.

86. Mason, C. and L.Erskine. 2000. Growth cone form, behavior, and interactions in vivo: retinal axon pathfinding as a model. *J. Neurobiol.* 44:260-270.

87. McConnachie, G., I.Pass, S.M.Walker, and C.P.Downes. 2003. Interfacial kinetic analysis of the tumour suppressor phosphatase, PTEN: evidence for activation by anionic phospholipids. *Biochem. J.* 371:947-955.

88. Medema, R.H., G.J.Kops, J.L.Bos, and B.M.Burgering. 2000. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* 404:782-787.

89. Mehenni, H., N.Lin-Marq, K.Buchet-Poyau, A.Reymond, M.A.Collart, D.Picard, and S.E.Antonarakis. 2005. LKB1 interacts with and phosphorylates PTEN: a functional link between two proteins involved in cancer predisposing syndromes. *Hum. Mol. Genet.* 14:2209-2219.

90. Mellor, H. 2004. Cell motility: Golgi signalling shapes up to ship out. *Curr. Biol.*R434-R435.

91. Millard, T.H. and L.M.Machesky. 2001. The Wiskott-Aldrich syndrome protein (WASP) family. *Trends Biochem. Sci.* 26:198-199.

92. Mitra, S.K., D.A.Hanson, and D.D.Schlaepfer. 2005. Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Biol.* 6:56-67.

93. Moon, S.K., H.M.Kim, and C.H.Kim. 2004. PTEN induces G1 cell cycle arrest and inhibits MMP-9 expression via the regulation of NF-kappaB and AP-1 in vascular smooth muscle cells. *Arch. Biochem. Biophys.* 421:267-276.

94. Mullins, R.D., J.A.Heuser, and T.D.Pollard. 1998. The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc. Natl. Acad. Sci. U. S. A.* 95:6181-6186.

95. Mullins, R.D., W.F.Stafford, and T.D.Pollard. 1997. Structure, subunit topology, and actin-binding activity of the Arp2/3 complex from Acanthamoeba. *J. Cell Biol.* 136:331-343.

96. Murray, D. and B.Honig. 2002. Electrostatic control of the membrane targeting of C2 domains. *Mol. Cell* 9:145-154.

97. Myers, M.P., I.Pass, I.H.Batty, K.J.Van der, J.P.Stolarov, B.A.Hemmings, M.H.Wigler, C.P.Downes, and N.K.Tonks. 1998. The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proc. Natl. Acad. Sci. U. S. A* 95:13513-13518.

98. Myers, M.P. and N.K.Tonks. 1997. PTEN: sometimes taking it off can be better than putting it on. *Am. J. Hum. Genet.* 61:1234-1238.

99. Nalefski, E.A. and A.C.Newton. 2001. Membrane binding kinetics of protein kinase C betaII mediated by the C2 domain. *Biochemistry.* 40:13216-13229.

100. Nathoo, N., A.Chahlavi, G.H.Barnett, and S.A.Toms. 2005. Pathobiology of brain metastases. *J. Clin. Pathol.* 58:237-242.

101. Nobes, C.D. and A.Hall. 1995. Rho, Rac, and cdc42 GTPases regulate the assenbly of multimolecular focal complexes associated with actin stress fibres, lamellipodia and filopodia. *Cell* 81:53-62.

102. Nobes, C.D. and A.Hall. 1999. Rho GTPases control polarity, protrusion and adhesion during cell movement. *J. Cell Biol.*1235-1244.

103. Nourry, C., S.G.Grant, and J.P.Borg. 2003. PDZ domain proteins: plug and play! *Sci. STKE.* 179:-RE7.

104. Ochs, H.D. and A.J.Thrasher. 2006. The Wiskott-Aldrich syndrome. *J. Allergy Clin. Immunol.* 117:725-738.

105. Okumura, K., M.Zhao, R.A.Depinho, F.B.Furnari, and W.K.Cavenee. 2005. Cellular transformation by the MSP58 oncogene is inhibited by its physical interaction with the PTEN tumor suppressor. *Proc. Natl. Acad. Sci. U. S. A* 102:2703-2706.

106. Ong, S.H., Y.R.Hadari, N.Gotoh, G.R.Guy, J.Schlessinger, and I.Lax. 2001. Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. *Proc. Natl. Acad. Sci. U. S. A.* 98:6074-6079.

107. Otterbein, L.R., P.Graceffa, and R.Dominguez. 2001. The crystal structure of uncomplexed actin in the ADP state. *Science.* 293:708-711.

108. Palanivelu, R. and D.Preuss. 2000. Pollen tube targeting and axon guidance: parallels in tip growth mechanisms. *Trends Cell Biol.* 10:517-524.

109. Park, K.C., F.Rivero, R.Meili, S.Lee, F.Apone, and R.A.Firtel. 2004. Rac regulation of chemotaxis and morphogenesis in Dictyostelium. *EMBO J.* 23:4177-4189.

110. Pepio, A.M. and W.S.Sossin. 2001. Membrane translocation of novel protein kinase Cs is regulated by phosphorylation of the C2 domain. *J. Biol. Chem.* 276:3846-3855.

111. Pizarro-Cerda, J. and P.Cossart. 2006. Subversion of cellular functions by Listeria monocytogenes. *J. Pathol.* 208:215-223.

112. Pollard, T.D. 1986. Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. *J. Cell Biol.* 103:2747-2754.

113. Pollard, T.D. and C.C.Beltzner. 2002. Structure and function of the Arp2/3 complex. *Curr. Opin. Struct. Biol.* 12:768-774.

114. Pollard, T.D., L.Blanchoin, and R.D.Mullins. 2000. Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu. Rev. Biophys. Biomol. Struct.* 29:545-76.:545-576.

115. Pring, M., A.Weber, and M.R.Bubb. 1992. Profilin-actin complexes directly elongate actin filaments at the barbed end. *Biochemistry.* 31:1827-1836.

116. Radu, A., V.Neubauer, T.Akagi, H.Hanafusa, and M.M.Georgescu. 2003. PTEN induces cell cycle arrest by decreasing the level and nuclear localization of cyclin D1. *Mol. Cell Biol.* 23:6139-6149.

117. Rafelski, S.M. and J.A.Theriot. 2004. Crawling toward a unified model of cell mobility: spatial and temporal regulation of actin dynamics. *Annu. Rev. Biochem.* 73:209-39.:209-239.

118. Raftopoulou, M. 2003. Role of Rho GTPases and PTEN in the Migration of Human Glioma Cells. *PhD Thesis, Univ. of London.*

119. Raftopoulou, M., S.Etienne-Manneville, A.Self, S.Nicholls, and A.Hall. 2004. Regulation of cell migration by the C2 domain of the tumor suppressor PTEN. *Science* 20;303:1179-1181.

120. Raftopoulou, M. and A.Hall. 2004. Cell migration: Rho GTPases lead the way. *Dev. Biol.* 265:23-32.

121. Ridley, A.J. and A.Hall. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell.* 70:389-399.

122. Ridley, A.J., M.A.Schwartz, K.Burridge, R.A.Firtel, M.H.Ginsberg, G.Borisy, J.T.Parsons, and A.R.Horwitz. 2003. Cell migration: integrating signals from front to back. *Science.* 302:1704-1709.

123. Robinson, R.C., K.Turbedsky, D.A.Kaiser, J.B.Marchand, H.N.Higgs, S.Choe, and T.D.Pollard. 2001. Crystal structure of Arp2/3 complex. *Science.* 294:1679-1684.

124. Salmeen, A. and D.Barford. 2005. Functions and mechanisms of redox regulation of cysteine-based phosphatases. *Antioxid. Redox. Signal.* 7:560-577.

125. Sarkar, D., H.Boukerche, Z.Z.Su, and P.B.Fisher. 2004. mda-9/syntenin: recent insights into a novel cell signaling and metastasis-associated gene. *Pharmacol. Ther.* 104:101-115.

126. Sauer, H. and M.Wartenberg. 2005. Reactive oxygen species as signaling molecules in cardiovascular differentiation of embryonic stem cells and tumor-induced angiogenesis. *Antioxid. Redox. Signal.* 7:1423-1434.

127. Schmidt, A. and A.Hall. 2002. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev.* 16:1587-1609.

128. Seelig, A., X.L.Blatter, A.Frentzel, and G.Isenberg. 2000. Phospholipid binding of synthetic talin peptides provides evidence for an intrinsic membrane anchor of talin. *J. Biol. Chem.* 275:17954-17961.

129. Shim, J.H., Y.S.Kim, and Y.Y.Bahk. 2006. Proteome profile changes that are differentially regulated by lipid and protein phosphatase activities of tumour suppressor PTEN in PTEN-expressing U-87 MG human glioblastoma cells. *Proteomics.* 6:81-93.

130. Shin, I., F.M.Yakes, F.Rojo, N.Y.Shin, A.V.Bakin, J.Baselga, and C.L.Arteaga. 2002. PKB/Akt mediates cell-cycle progression by

phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. *Nat. Med.* 8:1145-1152.

131. Smith, A., A.Smith, S.Alrubaie, C.Coehlo, S.J.Leevers, and A.Ashworth. 1999. Alternative splicing of the Drosophila PTEN gene. *Biochim. Biophys. Acta* 1447:313-317.

132. Stambolic, V., D.MacPherson, D.Sas, Y.Lin, B.Snow, Y.Jang, S.Benchimol, and T.W.Mak. 2001. Regulation of PTEN transcription by p53. *Mol. Cell* 8:317-325.

133. Stambolic, V., A.Suzuki, J.L.de la Pompa, G.M.Brothers, C.Mirtsos, T.Sasaki, J.Ruland, J.M.Penninger, D.P.Siderovski, and T.W.Mak. 1998. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 95:29-39.

134. Steck, P.A., M.A.Pershouse, S.A.Jasser, W.K.Yung, H.Lin, A.H.Ligon, L.A.Langford, M.L.Baumgard, T.Hattier, T.Davis, C.Frye, R.Hu, B.Swedlund, D.H.Teng, and S.V.Tavtigian. 1997. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.* 15:356-362.

135. Svitkina, T.M. and G.G.Borisy. 1999. Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. *J. Cell Biol.* 145:1009-1026.

136. Tadokoro, S., S.J.Shattil, K.Eto, V.Tai, R.C.Liddington, J.M.de Pereda, M.H.Ginsberg, and D.A.Calderwood. 2003. Talin binding to integrin beta tails: a final common step in integrin activation. *Science.* 302:103-106.

137. Takenawa, T. and H.Miki. 2001. WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. *J. Cell Sci.* 114:1801-1809.

138. Tamura, M., J.Gu, E.H.Danen, T.Takino, S.Miyamoto, and K.M.Yamada. 1999. PTEN interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway. *J. Biol. Chem.* 274:20693-20703.

139. Tamura, M., J.Gu, K.Matsumoto, S.Aota, R.Parsons, and K.M.Yamada. 1998. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science* 280:1614-1617.

140. Tapparel, C., A.Reymond, C.Girardet, L.Guillou, R.Lyle, C.Lamon, P.Hutter, and S.E.Antonarakis. 2003. The TPTE gene family: cellular expression, subcellular localization and alternative splicing. *Gene* 323:189-99.:189-199.

141. Thompson, J.D., T.J.Gibson, F.Plewniak, F.Jeanmougin, and D.G.Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876-4882.

142. Tolkacheva, T., M.Boddapati, A.Sanfiz, K.Tsuchida, A.C.Kimmelman, and A.M.Chan. 2001. Regulation of PTEN binding to MAGI-2 by two putative phosphorylation sites at threonine 382 and 383. *Cancer Res.* 61:4985-4989.

143. Tonks, N.K. and M.P.Myers. 1999. Structural assets of a tumor suppressor. *Science* 286:2096-2097.

144. Torres, J. and R.Pulido. 2001. The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. *J. Biol. Chem.* 276:993-998.

145. Torres, J., J.Rodriguez, M.P.Myers, M.Valiente, J.D.Graves, N.K.Tonks, and R.Pulido. 2003. Phosphorylation-regulated cleavage of the tumor suppressor PTEN by caspase-3: implications for the control of protein stability and PTEN-protein interactions. *J. Biol. Chem.* 278:30652-30660.

146. Valiente, M., A.ndres-Pons, B.Gomar, J.Torres, A.Gil, C.Tapparel, S.E.Antonarakis, and R.Pulido. 2005. Binding of PTEN to specific PDZ domains contributes to PTEN protein stability and phosphorylation by microtubule-associated serine/threonine kinases. *J. Biol. Chem.* 280:28936-28943.

147. Vanhaesebroeck, B., S.J.Leevers, K.Ahmadi, J.Timms, R.Katso, P.C.Driscoll, R.Woscholski, P.J.Parker, and M.D.Waterfield. 2001. Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* 70:535-602.:535-602.

148. Vanhaesebroeck, B. and M.D.Waterfield. 1999. Signaling by distinct classes of phosphoinositide 3-kinases. *Exp. Cell Res.* 253:239-254.

149. Vazquez, F., S.R.Grossman, Y.Takahashi, M.V.Rokas, N.Nakamura, and W.R.Sellers. 2001. Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. *J. Biol. Chem.* 276:48627-48630.

150. Vazquez, F., S.Ramaswamy, N.Nakamura, and W.R.Sellers. 2000. Phosphorylation of the PTEN tail regulates protein stability and function. *Mol. Cell Biol.* 20:5010-5018.

151. Viglietto, G., M.L.Motti, P.Bruni, R.M.Melillo, A.D'Alessio, D.Califano, F.Vinci, G.Chiappetta, P.Tsichlis, A.Bellacosa, A.Fusco, and M.Santoro.

2002. Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. *Nat. Med.* 8:1136-1144.

152. Virolle, T., E.D.Adamson, V.Baron, D.Birle, D.Mercola, T.Mustelin, and B.De, I. 2001. The Egr-1 transcription factor directly activates PTEN during irradiation-induced signalling. *Nat. Cell Biol.* 3:1124-1128.

153. Walker, S.M., C.P.Downes, and N.R.Leslie. 2001. TPIP: a novel phosphoinositide 3-phosphatase. *Biochem. J.* 360:277-283.

154. Walker, S.M., N.R.Leslie, N.M.Perera, I.H.Batty, and C.P.Downes. 2004. The tumour-suppressor function of PTEN requires an N-terminal lipid-binding motif. *Biochem. J.* 379:301-307.

155. Wang, P., Y.Wu, X.Ge, L.Ma, and G.Pei. 2003. Subcellular localization of beta-arrestins is determined by their intact N domain and the nuclear export signal at the C terminus. *J. Biol. Chem.* 278:11648-11653.

156. Ward, S.G. 2006. T lymphocytes on the move: chemokines, PI 3-kinase and beyond. *Trends Immunol.* 27:80-87.

157. Wear, M.A. and J.A.Cooper. 2004. Capping protein: new insights into mechanism and regulation. *Trends Biochem. Sci.* 29:418-428.

158. Wozniak, M.A., K.Modzelewska, L.Kwong, and P.J.Keely. 2004. Focal adhesion regulation of cell behaviour. *Biochim. Biophys. Acta* 1692:103-119.

159. Wu, X., K.Hepner, S.Castelino-Prabhu, D.Do, M.B.Kaye, X.J.Yuan, J.Wood, C.Ross, C.L.Sawyers, and Y.E.Whang. 2000a. Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2. *Proc. Natl. Acad. Sci. U. S. A* 97:4233-4238.

160. Wu, Y., D.Dowbenko, S.Spencer, R.Laura, J.Lee, Q.Gu, and L.A.Lasky. 2000b. Interaction of the tumor suppressor PTEN/MMAC with a PDZ domain of MAGI3, a novel membrane-associated guanylate kinase. *J. Biol. Chem.* 275:21477-21485.

161. Wymann, M.P. and R.Marone. 2005. Phosphoinositide 3-kinase in disease: timing, location, and scaffolding. *Curr. Opin. Cell Biol.* 17:141-149.

162. Yamada, K.M. and M.Araki. 2001. Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. *J. Cell Sci.* 114:2375-2382.

163. Yamashita, A., K.Maeda, and Y.Maeda. 2003. Crystal structure of CapZ: structural basis for actin filament barbed end capping. *EMBO J.* 22:1529-1538.

164. Yu, J.W. and M.A.Lemmon. 2001. All Phox Homology (PX) Domains from *Saccharomyces cerevisiae*
Specifically Recognize Phosphatidylinositol 3-Phosphate. *J. Biol. Chem.* 276:44179-44184.

165. Zaidel-Bar, R., C.Ballestrem, Z.Kam, and B.Geiger. 2003. Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. *J. Cell Sci.* 116:4605-4613.

166. Zhang, D., M.Bar-Eli, S.Meloche, and P.Brodt. 2004. Dual regulation of MMP-2 expression by the type 1 insulin-like growth factor receptor: the phosphatidylinositol 3-kinase/Akt and Raf/ERK pathways transmit opposing signals. *J. Biol. Chem.* 279:19683-19690.

167. Zhang, D. and P.Brodt. 2003. Type 1 insulin-like growth factor regulates MT1-MMP synthesis and tumor invasion via PI 3-kinase/Akt signaling. *Oncogene* 20;22:974-982.

168. Zmijewski, J.W., A.Landar, N.Watanabe, D.A.Dickinson, N.Noguchi, and V.M.rley-Usmar. 2005. Cell signalling by oxidized lipids and the role of reactive oxygen species in the endothelium. *Biochem. Soc. Trans.* 33:1385-1389.